

**Whole genome amplification for PGD and PND;  
molecular and a-CGH diagnosis**

**By**

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## **Declaration**

I, Stavros Glentis, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.



**I dedicate this work to my Father**

## **Acknowledgements**

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## **Abstract**

Whole genome amplification amplifies the entire genome in a few hours from samples of minimal DNA quantities, even from single cells. This may have many applications, especially in prenatal diagnosis, PGD and PGS.

The hypothesis for chapter 3 was: Can multiple displacement amplification (MDA) be used as a universal step prior to molecular analysis for PGD? WGA using MDA (Qiagen) was used on single cells in order to overcome the problem of limited DNA in PGD. MDA allows the diagnosis through haplotyping or a combination of direct and indirect mutation analysis. Different cell types, including buccal cells, lymphocytes, fibroblasts and blastomeres were examined. A modification on the cell lysis buffer was also tested in order to achieve more accurate results. PGD seems to benefit from MDA when multiple tests are performed for direct and indirect analysis. The modified lysis buffer (exclusion of DTT) produced better results than the other lysis buffers and buccal cells do not produce as accurate results as other cell types. The hypothesis was met as the amount of DNA produced by MDA can be used for direct and indirect testing and haplotyping.

The hypothesis for chapter 4 was: Is it possible to accurately assess the chromosomes of a single cell by a-CGH? WGA was achieved by MDA and GenomePlex (Sigma) on single lymphocytes, fibroblasts and blastomeres prior to a-CGH analysis. The difficulty of this technique was the high background noise that was produced by WGA that makes interpretation difficult. Different lysis buffers, modifications of the WGA reaction and analysis software were examined for better results. A-CGH slides from different companies and institutions were used. The results showed that GenomePlex produced less background noise compared to MDA but the amplification efficiency of the technique was less reliable. The BlueGnome Cytochip arrays produced the best compared to arrays from any other companies or institutions. More experiments would be necessary to determine if the hypothesis was met as a number of chromosomal abnormalities detected were not always confirmed by other experiments.

The hypothesis for chapter 5 was: Can aneuploidy be detected in coelomic fluid using a-CGH? The possibility of using WGA and a-CGH on coelomic fluid was tested as this could be used as an early form of prenatal diagnosis. Coelomic fluid was collected between the 5<sup>th</sup> and 11<sup>th</sup> week of pregnancy from women undergoing termination of pregnancy. MDA and GenomePlex were used to amplify the DNA prior to a-CGH analysis. Both genomic (high resolution) and constitutional (low resolution) arrays were tested. The results showed that aneuploidy can be detected by a-CGH. BlueGnome Cytochip slides produced the best results. A triploid sample was detected as normal. The hypothesis was met and even higher resolution could be achieved with the use of GenomePlex and BlueGnome Cytochip arrays.

WGA may be very important for downstream genetic tests when the DNA is from very low quality and quantity. Further optimisation of the technique is needed in order to achieve similar results to those of good quality genomic DNA. Arrays from different companies or institutions may produce very different results. In conclusion, the results showed that WGA can benefit PGD and PND, and a-CGH gives great potential to PGS and coelomic fluid diagnosis.

## Table of contents

<b>Title</b>	<b>1</b>
<b>Declaration</b>	<b>2</b>
<b>Dedication</b>	<b>3</b>
<b>Acknowledgments</b>	<b>4</b>
<b>Abstract</b>	<b>5</b>
<b>Table of contents</b>	<b>7</b>
<b>List of tables</b>	<b>11</b>
<b>List of figures</b>	<b>12</b>
<b>List of abbreviations</b>	<b>14</b>
 <b>Chapter 1: Introduction</b>	 <b>15-47</b>
1.1 Genetic testing	15
1.1.1 Meiosis	15
1.1.2 Fertilisation and preimplantation development	16
1.1.3 Assisted reproduction technology	17
1.2 Preimplantation Genetic Diagnosis	20
1.3 Preimplantation Genetic Screening	22
1.4 Biopsy methods	23
1.5 Diagnosis techniques	24
1.5.1 Polymerase Chain Reaction	24
1.5.1.1 Contamination	24
1.5.1.2 Allele drop out	25
1.5.1.3 Total amplification failure	27
1.5.2 Whole genome amplification	27
1.5.2.1 PCR based WGA techniques	28
1.5.2.2 The Phi29 Polymerase	31
1.5.2.3 Multiple displacement amplification	33
1.5.3 WGA limitations	35
1.5.4 Preimplantation genetic haplotyping	37
1.5.5 Fluorescent in situ hybridisation	37
1.5.6 Metaphase comparative genomic hybridisation	38
1.5.7 Array comparative genomic hybridisation	39
1.5.7.1 Probes	40
1.5.7.2 Microarray Platforms	41
1.5.7.3 Fabrication	42
1.5.7.4 DNA sample labelling	43
1.6 Prenatal diagnosis and screening	43
1.7 Aims	44
 <b>Chapter 2: Materials and Methods</b>	 <b>48-55</b>
2.1 General	48
2.2.1 DNA extraction from blood	48
2.2.2 Culturing, collection and DNA extraction from skin fibroblasts	49

2.2.3	Lymphocyte and buccal cells collection	49
2.3	Single cell isolation and cell lysis	50
2.4	Whole genome amplification	52
2.4.1	MDA	52
2.4.2	GenomePlex	54
<b>Chapter 3: Molecular analysis of MDA products from single cells</b>		<b>56-102</b>
3.1	Hypothesis	56
3.2	Introduction	56
3.3	Aims	59
3.4	Experimental Design	60
3.5	Materials and methods	61
3.5.1	DNA materials	61
3.5.2	DNA concentrations	62
3.5.3	Ethical approval	63
3.5.4	Polymerase chain reaction	63
3.5.4.1	DM triplex	64
3.5.4.2	CF triplex	64
3.5.5	PCR analysis	65
3.5.6	Statistical analysis	65
3.6	Results	66
3.6.1	Preliminary experiments	66
3.6.1.1	DNA concentration	66
3.6.1.2	Allele sizes	66
3.6.1.3	Negative and positive controls for MDA	67
3.6.1.4	Single cell PCR	68
3.6.1.5	MDA using the L3 method	68
3.6.1.6	MDA using the L1 and L2 methods	72
3.6.2	Main results	78
3.6.2.1	MDA using L1 lysis - Comparison of different cell types	78
3.6.2.2	Combined results of the triplex reaction.	86
3.6.3	Qualitative analysis	87
3.6.3.1	MDA effect on stutter bands, preferential amplification and ADO	87
3.6.3.2	Comparison of 25 and 40 PCR cycles on MDA products	90
3.7	Discussion	92
3.7.1	Cell lysis	92
3.7.2	Cell types	95
3.7.3	MDA-AE	96
3.7.4	TAF and ADO	97
3.7.5	Inaccurate alleles, artefacts and bleed-through	98
3.7.6	Conclusion	100
<b>Chapter 4: Analysis of single cells by a-CGH</b>		<b>103-158</b>
4.1	Hypothesis	103

4.2	Introduction	103
4.3	Aims	104
4.4	Experimental Design	105
4.5	Materials and Methods	106
4.5.1	Ethical approval	106
4.5.2	Materials and DNA samples for a-CGH	106
4.5.3	Cells and DNA treatment before a-CGH	106
4.5.4	A-CGH	109
4.5.4.1	Sanger Institute	109
4.5.4.2	Spectral Genomics	109
4.5.4.3	Array Genomics	110
4.5.4.4	Translational Research Laboratory	111
4.5.4.5	BlueGnome	113
4.5.4.6	Scanning and analysis	115
4.5.5	FISH experiments	115
4.5.5.1	Slide preparation for skin fibroblast and lymphocytes	116
4.5.5.2	Slide preparation for human embryos	116
4.5.5.3	Slide pre-treatment	117
4.5.5.4	Probe preparation	117
4.5.5.5	Separate denaturation, hybridisation and post-hybridisation washes	118
4.5.5.6	Co-denaturation, hybridisation and post-hybridisation washes	118
4.5.5.7	Image analysis	119
4.5.5.8	Re-probing of embryos	119
4.6	Results	119
4.6.1	DNA and WGA products concentration and quality validation	119
4.6.2	Results from Sanger Institute a-CGH slides	122
4.6.3	A-CGH results with gDNA and MDA products from gDNA	124
4.6.4	A-CGH results with MDA products from single lymphocytes and fibroblasts	134
4.6.5	A-CGH results with MDA products from single blastomeres	142
4.7	Discussion	150
4.7.1	A-CGH platforms	151
4.7.2	WGA method for test and reference DNA	153
4.7.3	Analysis method and maximum resolution	155
4.7.4	Conclusion	157
<b>Chapter 5: A-CGH analysis of coelomic fluid</b>		<b>159-200</b>
5.1	Hypothesis	159
5.2	Introduction	159
5.3	Aims	160
5.4	Experimental design	161
5.5	Materials and Methods	164
5.5.1	Ethical approval	164
5.5.2	Coelocentesis and tissues collection	164
5.5.3	DNA extraction	164

5.5.4	WGA, PCR and DNA concentrations	165
5.5.5	DNA and tissue materials collected	165
5.5.6	Fluorescent in Situ Hybridisation (FISH)	166
5.5.6.1	Touch preparation	166
5.5.6.2	Probe preparation	166
5.5.6.3	FISH methods	167
5.5.7	Molecular cytogenetic analysis by a-CGH	167
5.6	Results	167
5.6.1	Molecular analysis for contamination	167
5.6.2	FISH analysis	170
5.6.3	DNA concentration	171
5.6.4	A-CGH analysis	173
5.6.4.1	A-CGH analysis of CoF 4	173
5.6.4.2	A-CGH analysis of CoF 7	176
5.6.4.3	A-CGH analysis of CoF 8	178
5.6.4.4	A-CGH analysis of CoF 10	181
5.6.4.5	A-CGH analysis of CoF 11	190
5.7	Discussion	195
5.7.1	Concentration CoF DNA and minimum amount for WGA	196
5.7.2	Different WGA methods for CoF DNA amplification	197
5.7.3	Molecular cytogenetic analysis of CoF by a-CGH	198
5.7.4	Conclusion	200
<b>Chapter 6: Conclusions</b>		201-207
6.1	Diagnosis with single cells and small numbers of cells	201
6.2	WGA for single cells and small numbers of cells	201
6.3	A-CGH platforms and analysis	203
6.4	Future experiments	206
<b>References</b>		209-232
<b>Appendix</b>		233-239
A.1	Suppliers of chemicals	233
A.2	Solutions for DNA extraction from blood	233
A.3	Solutions for DNA extraction from coelomic fluid	233
A.4	Materials and solutions for single cell isolation and lysis	234
A.5	Materials and solutions for WGA by MDA and GenomePlex	235
A.6	Materials and solutions for PCR	235
A.7	Materials and solutions for a-CGH	235
A.8	Materials and solutions for FISH	235
A.9	Agarose gel electrophoresis	237
A.10	Publications and abstracts arising from this thesis	239
<b>Publications</b>		240-261



## List of tables

### Chapter 2

Table 2.1	Summary of different lysis methods used for single cell lysis	52
Table 2.2	Protocols of DNA source and modifications of the WGA techniques.	53

### Chapter 3

Table 3.1	Summary of DNA source used.	64
Table 3.2	Details of the primers used for chapter 3	65
Table 3.3	True allele sizes after PCR of gDNA.	69
Table 3.4	Triplex PCR on single buccal cells and lymphocytes after L1 or L3 lysis.	71
Table 3.5	Summary of PCR analysis of single cells lysed with the L1 and L3 methods.	72
Table 3.6.	Triplex PCR on MDA products from single buccal cells and lymphocytes after L3 lysis.	74
Table 3.7	PCR on MDA products from single buccal cells and lymphocytes after L1 and L2 lysis.	75
Table 3.8	Summary of PCR analysis on MDA products lysed with L1 and L2 methods	76
Table 3.9	Comparison of different cell types with L1 lysis method.	82
Table 3.10	Summary of the results of single cell MDA products from different cell types with L1 lysis.	84
Table 3.11	Summary of the results from MDA products from single blastomeres and comparison with other cell types.	85

### Chapter 4

Table 4.1	Summary of probe mixes used for FISH on fibroblasts and embryos.	119
Table 4.2	DNA samples prepared for the array experiments.	123
Table 4.3	PCR validation of MDA reaction with PCR.	124
Table 4.4	Sanger Institute a-CGH experiments.	125
Table 4.5	A-CGH experiments with gDNA and WGA products from gDNA.	126
Table 4.6	Experiments performed with WGA products from single lymphocytes and fibroblasts.	136
Table 4.7	PCR analysis with 10 markers on five chromosomes for blastomeres.	146
Table 4.8	Summary of results obtained from WGA products from single blastomeres	152

### Chapter 5

Table 5.1	Details of the coelomic fluid and other materials collected.	167
Table 5.2	Summary of probe mixes used for the prenatal project.	168
Table 5.3	Allele sizes of CoF samples and maternal DNA.	171
Table 5.4	Fluorescent signals for chromosomes X, Y and 18 from the placenta tissue samples:	172
Table 5.5	DNA concentration of CoF samples and their WGA products.	174
Table 5.6	Summary of results of the a-CGH with CoF 4.	178

Table 5.7	Summary of results of the a-CGH with CoF 7.	178
Table 5.8	Summary of results of the a-CGH with CoF 8.	183
Table 5.9	Summary of results of the a-CGH with CoF 10 MDA products.	187
Table 5.10	Summary of results of the a-CGH with CoF 10 MDA and GenomePlex products.	189
Table 5.11	Summary of results of the a-CGH with CoF 11 WGA products.	192
Table 5.12	Summary of a-CGH for this chapter.	196

## **Appendix**

Table A1	PCR primer sequences.	240
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## **List of figures**

### **Chapter 1**

Figure 1.1	Spermatogenesis and oogenesis	18
Figure 1.2	Fertilisation and preimplantation development	19
Figure 1.3	ICSI	20
Figure 1.4	Diagram of GenomePlex WGA method:	30
Figure 1.5	Structure of Phi29 polymerase with terminal protein	33
Figure 1.6	Diagram of MDA.	34

### **Chapter 3**

Figure 3.1	Location of markers on chromosomes 19 and 7.	61
Figure 3.2	Synopsis of the experimental design of this chapter.	62
Figure 3.3	Electropherograms of single cell PCR for the DM triplex	75
Figure 3.4	Electropherograms of single cell MDA products after L3 lysis	76
Figure 3.5	Electropherograms of single cell MDA products after L1 lysis	78
Figure 3.6	Electropherograms of single cell MDA products after L2 lysis	79
Figure 3.7	Electropherograms of single fibroblasts MDA products after L1 lysis.	86
Figure 3.8	Electropherograms of single blastomeres MDA products after L1 lysis.	87
Figure 3.9	Efficiency of diagnosis for DM triplex from different cell types with and without MDA.	88
Figure 3.10	Qualitative analysis of MDA from gDNA.	90
Figure 3.11	Comparison of PCR results of MDA products from different cell types with gDNA.	90
Figure 3.12	Electropherogram from MDA buccal cells and gDNA for the D19S112 locus	91
Figure 3.13	Presence of inaccurate allele and ADO.	91
Figure 3.14	Presence of inaccurate allele size.	92
Figure 3.15	Comparison of results of the same MDA product after different PCR cycles.	93

Figure 3.16	Extreme presence of artefacts in more PCR cycles.	94
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## **Chapter 4**

Figure 4.1	Experimental design of the single cell a-CGH experiments.	109
Figure 4.2	Graphs of the result from experiment A1.	125
Figure 4.3	Spectral Genomics 2.0™ experiment B1 Ideograms.	127
Figure 4.4	Spectral Genomics 3.0™ experiment B2 Ideograms.	128
Figure 4.5	Array Genomics experiment 2 ideograms.	130
Figure 4.6	Fibroblast FISH analysis.	131
Figure 4.7	TRL Mermaid arrays experiment B5.	132
Figure 4.8	Cytochip prenatal ideograms of a-CGH experiment B6.	134
Figure 4.9	Cytochip 1MB ideograms from experiment B7 (MDA product from gDNA).	135
Figure 4.10	Spectral Genomics constitutional 2.0™ experiment C1.	137
Figure 4.11	Array Genomics prenatal slide result of experiment C3.	138
Figure 4.12	TRL Mermaid array slides of experiment C4.	139
Figure 4.13	BlueGnome Cytochip 1MB slide of experiment C6.	141
Figure 4.14	BlueGnome Cytochip prenatal slide Ideograms of experiment C7 (MDA P2 protocol).	142
Figure 4.15	BlueGnome Cytochip prenatal results of experiments C8 and C9 (GenomePlex P6 and P7 protocols).	143
Figure 4.16	BlueGnome Cytochip 1MB slide result of experiment D1 (Embryo 1, blastomere 1, MDA).	145
Figure 4.17	BlueGnome Cytochip 1MB slide result of experiment D2 (Embryo 2, blastomere 1, MDA).	148
Figure 4.18	BlueGnome Cytochip 1MB slide result of experiment D3 (Embryo 2, blastomere 2, GenomePlex).	149
Figure 4.19	BlueGnome Cytochip 1MB slide result of experiment D4 (Embryo 3, blastomere 4, MDA).	151

## **Chapter 5**

Figure 5.1	Summary of experimental design for the coelomic fluid study.	164
Figure 5.2	FISH analysis of placenta samples for chromosomes 18, X and Y.	175
Figure 5.3	A-CGH analysis of CoF 4 MDA products.	176
Figure 5.4	A-CGH analysis of CoF 4 GenomePlex products.	177
Figure 5.5	A-CGH analysis of CoF 7 MDA and GenomePlex products.	179
Figure 5.6	Ideograms of Chromosome X from a-CGH experiments with CoF 7.	180
Figure 5.7	A-CGH analysis of CoF 8 GenomePlex products with Cytochip prenatal and Mermaid 32K slides.	181
Figure 5.8	A-CGH analysis of CoF 8 GenomePlex and MDA products Spectral Genomics constitutional slides.	182
Figure 5.9	A-CGH analysis of CoF 10 MDA products with Cytochip 1MB slides	185
Figure 5.10	A-CGH analysis of CoF 10 MDA products with 32K Mermaid slides.	186

Figure 5.11	A-CGH analysis of CoF 10 GenomePlex products with the Cytochip prenatal slides.	188
Figure 5.12	A-CGH analysis of CoF 10 MDA product from 2.5µl of DNA with the Cytochip 1MB slide.	190
Figure 5.13	A-CGH analysis of MDA product derive from direct application on 5µl of CoF	191
Figure 5.14	A-CGH analysis of CoF 11 GenomePlex and MDA products with Cytochip prenatal slides.	193
Figure 5.15	FISH analysis of placenta CoF 11 for chromosomes 13, 15 and 21.	194
Figure 5.16	A-CGH analysis of CoF 11 GenomePlex product with 32K Mermaid slide.	195

### List of abbreviations

A-CGH	Array comparative genomic hybridisation
ADO	Allele drop out
BAC	Bacterial artificial chromosome
CF	Cystic fibrosis
cfDNA	Cell free DNA
CNV	Copy number variant
CoF	Coelomic fluid
DM	Dystrophia Myotonia
DOP-PCR	Degenerate oligonucleotide primed-PCR
DTT	Dithiothreitol
EAS	Expected allele size
ERPC	Evacuation of retained products of conception
F-PCR	Fluorescent polymerase chain reaction
FISH	Fluorescent in situ hybridisation
FFPE	Formalin-fixed paraffin-embedded
IVF	In vitro fertilization
LA-PCR	Linker adaptor PCR
M-CGH	Metaphase comparative genomic hybridisation
MDA	Multiple displacement amplification
MDA-AE	MDA- amplification efficiency
OD	Optical density
PEP	Primer extension preamplification
PGD	Preimplantation genetic diagnosis
PGH	Preimplantation genetic haplotyping
PGS	Preimplantation genetic screening
PND	Prenatal diagnosis
STR	Short tandem repeat
TAF	Total amplification failure
TOP	Termination of pregnancy
WGA	Whole genome amplification

# **Chapter 1**

## **Introduction**

### **1.1 Genetic testing**

DNA is the fundamental substance for every living organism which is passed from generation to generation. Abnormal DNA is responsible for certain diseases and defects which are known as genetic disorders. The evolution in the field of genetics has allowed the identification of genetic abnormalities even before the symptoms appear. Specific genetic tests can be performed focusing at the molecular or chromosomal level after obtaining DNA from the individual. In many cases of genetic diseases there is no treatment. In such cases the avoidance of transmitting this harmful genetic disease to the next generations is essential. The earliest stage that a genetic test can be performed is at the 8-cell stage in which the test can predict if the developing organism will develop a specific disorder.

#### **1.1.1 Meiosis**

A genetic abnormality can be transmitted from one generation to the other as an inherited mutation, can be a novel mutation in a somatic cell (like cancer) or can arise during the very complex mechanism of meiosis of the gametes or in the first cell divisions (post-zygotic errors). In animals meiosis involves a process in which specific cells follow two consecutive cell divisions in order to produce haploid cells, the gametes (Strachan and Read, 1999). The gametes in humans are the spermatozoa that are produced in the male reproductive system (testis) and the oocytes that are produced in the female reproductive system (ovary). The production of spermatozoa (spermatogenesis) and oocytes (oogenesis) is a very complex mechanism. Spermatogenesis takes place in the testis where spermatogonia cells (primordial germ cells that have entered the testis) proliferate by mitotic division until they differentiate to primary spermatocytes (diploid cell) (Gilbert, 2001). Primary spermatocytes enter meiosis which will produce four mature spermatozoa. In meiosis I, the DNA is compacted to 46 chromosomes (23 pairs of

homologous chromosomes) that are aligned in pairs in the centre of the cell. Crossing over takes place and the first meiotic division is completed with the production of two cells (secondary spermatocytes) where each contains one set of homologous chromosomes with the two sister chromatids connected at the centromere. In meiosis II, the centromeres divide and the sister chromatids are separated apart resulting in four cells (spermatids) from the two secondary spermatocytes. The spermatids will mature and differentiate into spermatozoa (Figure 1). Spermatogenesis is a process that happens from puberty throughout the adult life of male organisms (Mueller and Young, 2001).

In contrast to spermatogenesis, oogenesis does not happen from puberty throughout the adult life of the females. Oogenesis starts from the first months of the embryonic life where oogonia (primordial germ cells that have entered the ovaries) proliferate and develop to primary oocytes which will start the meiosis. At birth, the primary oocytes have entered a phase of maturation arrest (dictyotene stage) in which they remain suspended until puberty (Strachan and Read, 1999). Meiosis I is completed in the follicle before ovulation, where a single secondary oocyte and a polar body is formed. Unlike spermatogenesis, the secondary oocyte receives the majority of the cytoplasm compared to the polar body. The secondary oocyte enters meiosis II stage but arrests at the metaphase II stage. Meiosis II will only be completed if fertilisation occurs after the oocyte is being released by the follicle in the oviduct (figure 1). In case of fertilisation a second polar body will be formed. As meiosis in females can take up to 50 years to be completed, there is an increased risk of chromosomal abnormalities connected with maternal age related to meiosis errors (Mueller and Young, 2001).

### **1.1.2 Fertilisation and preimplantation development**

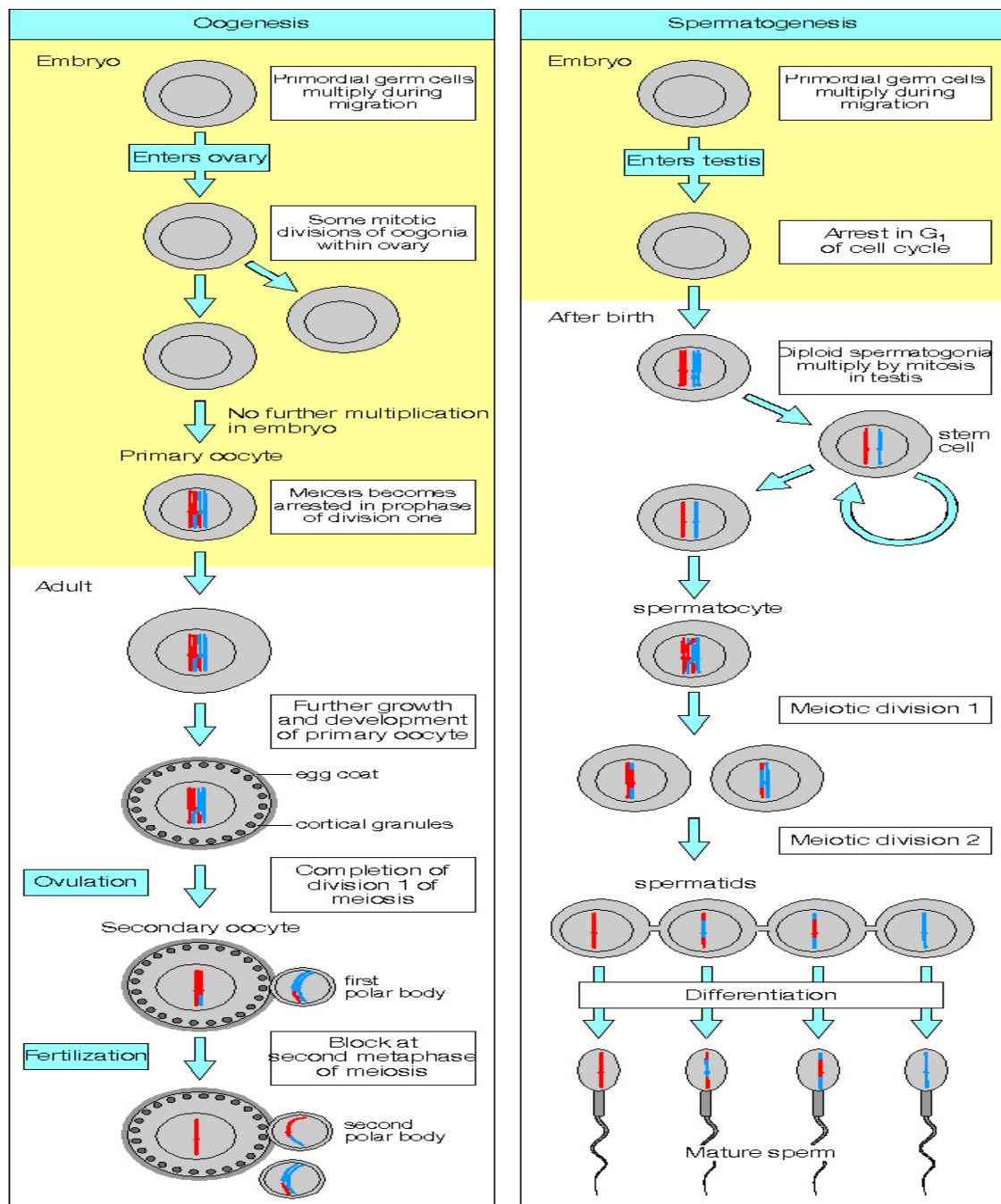
Fertilisation occurs when the male and female gametes fuse and produce a new organism. Fertilisation takes place in the ampulla of the oviduct, a region close to the ovary. In humans, millions of spermatozoa will surround the female gamete but only one will manage to drill the zona pellucida and release the sperm DNA inside the oocyte. After fertilisation, the first mitotic division of the new organism (cleavage) begins in the next

24 hours while the new organism moves towards the uterus. After the first cleavage, DNA transcription also begins and maternal mRNA degrades. The two cells divide asynchronously until they reach eight cells in the following two days. Until that stage the cells have a loose arrangement with space between them (cleavage stage). After that stage, the cells form tight junctions with each other and maximizing their contact and become a compact ball of cells (phenomenon of compaction). Asynchronous mitotic division continues and in the following days the embryo moves from the morula stage to the blastocyst stage, where differentiation of the cells occurs. The cells located in the inner core form the inner cell mass (ICM) are the ones that will give rise to all tissues of the embryo body and some extraembryonic tissues (amnion, yolk sack and allantois) whereas the cells in the outer core form the trophoctoderm which is responsible for the production of the placenta (Gilbert, 2001).. As the blastocyst is growing and dividing, it reaches the uterus on day 6-7, removes the zona pellucida (hatching) and it is finally implanted at the uterus between days 8 and 9 (figure 2).

### **1.1.3 Assisted reproduction technology**

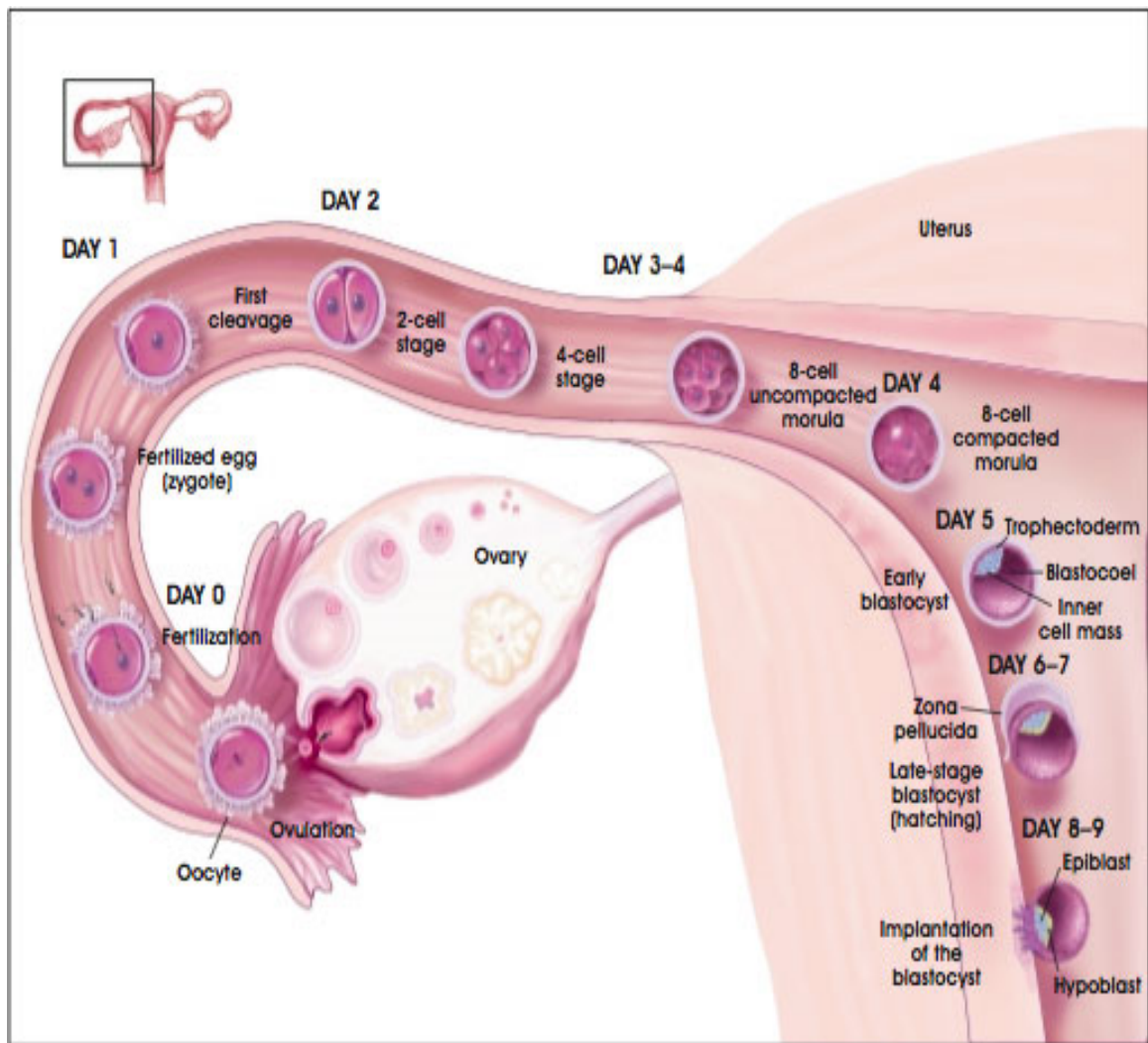
Assisted reproduction technology (ART) refers to methods used in order to achieve a pregnancy that are usually used in couples with fertility problems. Fertility problems can be present in males and females. Common infertility problems of males is azoospermia (complete absence of sperm in the semen) and oligospermia (<20 million sperms/ ml per ejaculation produced) which can be due to hormonal problems, environmental factors, infections or chromosomal abnormalities. In females infertility problems can be related with the oocyte (chromosomal abnormalities, advanced maternal age) or with the ovulation (mainly hormonal problems) or with the anatomy of the female genital tract (like endometriosis). The most common ART is in vitro fertilisation (IVF) in which the oocyte is fertilised *in vitro* by spermatozoa outside the womb (Mueller and Young, 2001). Different techniques have been developed for IVF, including intra-cytoplasmic sperm injection (ICSI) where a single sperm is injected in the oocyte via a microneedle (figure 1.3) and assisted zona hatching (AZH) where a small opening of the zona pellucida is made prior to embryo transfer that helps the embryo in the implantation process in the

uterus. IVF embryos can also be biopsied and chromosomal and molecular diagnosis can be performed in order to avoid genetic abnormalities.

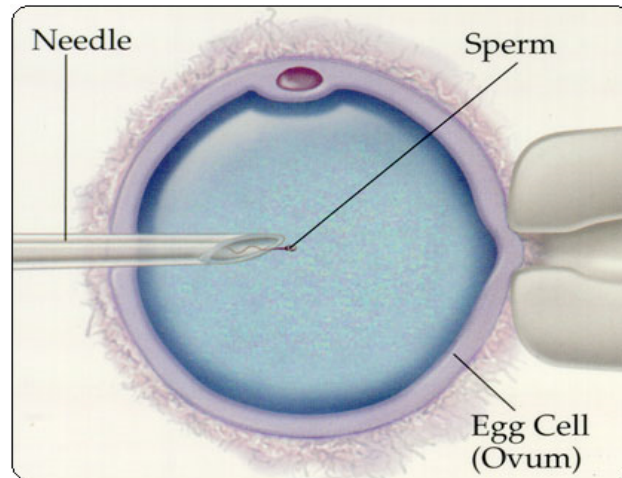


**Figure 1.1: Spermatogenesis and oogenesis.** This figure gives a schematic representation of how female and male primordial germ cells develop and divide to produce oocytes and spermatozoa respectively (figure adapted from Memorial University, Canada, <http://www.mun.ca/biology>).





**Figure 1.2: Fertilisation and preimplantation development.** This diagram shows all the stages from ovulation of the oocyte, to fertilisation, preimplantation development and final implantation at the uterus after eight to nine days (figure adopted from the National Institutes of health resource for stem cell research, <http://stemcells.nih.gov>).



**Figure 1.3: ICSI.** This diagram shows how ICSI is performed. A microneedle that contains a single sperm is injecting the oocyte and releases the sperm in order to assist fertilisation. (Figure adopted from Colorado Reproductive endocrinology website, [www.coloradofertility.com](http://www.coloradofertility.com))

## 1.2 Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis (PGD) involves the genetic analysis of cells from an oocyte or embryo for a specific molecular mutation or chromosomal abnormality (Fragouli, 2007). The purpose of PGD is to determine which embryos that are generated by IVF are normal for the specific genes and chromosomes for which they are assessed so that these can be selected for implantation (Wells, 2004). The PGD tests that are being performed due to single gene diseases use the polymerase chain reaction (PCR) technique whereas PGD tests for chromosomal abnormalities use fluorescent in situ hybridisation (FISH) analysis of single blastomeres (Harper et al., 2008a).

Regarding single gene diseases, more than two hundred have been tested in clinical PGD (Fragouli et al., 2007). In theory, every known single gene mutation could be diagnosed at a single cell (Spits et al., 2009). However, in practice, PGD-PCR protocols can be very difficult to be optimised, due to the limited amount of DNA present for diagnosis. The most common PGD tests for single gene defects used are cystic fibrosis, beta thalassaemia, myotonic dystrophy, Huntington's disease and fragile X syndrome (Spits et

al., 2009; Fragouli et al., 2007). The same monogenic disorders are the most commonly tested in prenatal diagnosis. In PGD however, there is also an increasing number of diseases that are not offered in prenatal diagnosis; these are late-onset diseases (such as cancer), human leukocyte antigen (HLA) testing and predisposition syndromes (Goosens et al., 2008).

Fluorescent PCR (F-PCR) is most commonly used in PGD-PCR protocols as it is over a thousand times more sensitive than the PCR analysis on ethidium bromide stained gels. According to the PGD protocol, other post PCR methods can be used, including the use of restriction endonucleases leading to restriction fragment length polymorphism (RFLP), minisequencing, quantitative real-time PCR and amplification refractory mutation system (ARMS) (Spits et al., 2009; Fiorentino et al., 2003; Pierce et al., 2003).

Initial experiments for PGD were performed in mice (Monk et al., 1987). The first clinical PGD cycles were reported in 1990 in which PCR amplified a repeat sequence on the long arm of chromosome Y that was used to distinguish male from female embryos in families that were carriers of an X-linked recessive disease (Handyside et al., 1990). Since then, many single gene disorders have been diagnosed with the use of PCR in PGD (Harper et al., 2006). Interphase FISH substituted PCR for X-linked recessive diseases in 1993, as it was considered to be a more robust and efficient method (Delhanty et al., 1993). The technique evolved very rapidly in the PGD field and now is used to for a variety of chromosomal aberrations with the majority being reciprocal translocations (Harper et al., 2008b).

Couples that may undertake PGD are those that are at risk of transmitting a recessive, dominant or X-linked single gene disorder that is lethal, seriously debilitating, life threatening later in life or carriers of structural chromosomal abnormalities. Such couples may be against terminating an affected pregnancy because of ethical or religious reasons. PGD can also be carried out for predisposition syndromes and HLA-antigen matching (Wells, 2004).

### **1.3 Preimplantation genetic screening**

Preimplantation genetic screening (PGS) is a genetic test that uses PGD technology, but with a different purpose (Twisk et al., 2006). PGS is used to screen for chromosomal aneuploidies in IVF embryos. In theory, aneuploidy screening of the blastomeres or polar bodies would help to implant only the chromosomally normal embryos and this would lead to improved pregnancy rates after IVF and decreased miscarriage.

PGS has been applied widely by many clinics to IVF patients but controversial data have been published regarding if IVF benefits from PGS and in which IVF groups should be applied (Donoso et al., 2007). Some groups have concluded that PGS should not be applied to any IVF patient (Mastenbroek et al., 2007) whereas others recommended that PGS should be part to every IVF cycle (Kuliev et al., 2003). Universal application of PGS in IVF patients should be mathematically flawed due to false negative diagnostic rates (1.2-4.7% as suggested from Munne et al., 2005) and due to existence of mosaicism and self- correction of preimplantation stage embryos. On the other hand, excluding PGS for all IVF patient groups should not be concluded due to the different techniques (use of one or two blastomeres or polar body), laboratory performance and varying degrees of embryo manipulation used in different clinics. Most of the studies however conclude that IVF benefits from PGS when applied in certain groups of IVF patients (Staessen et al., 2008). The indications for PGS includes advance maternal age (Staessen et al., 2008), repeated implantation failure, repeated early miscarriage and severe male infertility (Harper et al., 2009; Twisk et al., 2006).

The vast majority of PGS tests involve FISH for five to 15 chromosomes whereas in rare cases metaphase comparative genomic hybridisation (m-CGH) has been applied in polar bodies and single blastomeres in which all chromosomes are screened (Baart et al., 2007; Wilton et al., 2003; Wells et al., 2002). Although m-CGH has technical limitations due to the extensive time needed for diagnosis, very recently microarray CGH (a-CGH) has started gaining ground in PGS analysis and the first clinical case of PGS with the use of a-CGH was reported (Hellani et al., 2008). Following that a number of pre-clinical PGS

studies with the use of a-CGH have been published providing very promising results for future use of the technique.

#### **1.4 Biopsy methods**

Polar-body, cleavage-stage or blastocyst biopsy has to be performed prior to PGD or PGS. Each of the biopsy methods has advantages and limitations (Sermon et al., 2004). In polar-body (PB) biopsy, polar bodies are aspirated after a small cut is made in the zona pellucida by sharp needles or laser (Sermon et al., 2004). This technique is useful only for the genotype analysis of the oocyte and not the embryo.

In cleavage-stage biopsy, the embryos are grown in vitro after fertilisation in an appropriate environment until they reach the 6- to 8-cell stage which occurs on the third day after insemination. In order to remove one or two cells from the embryo the zona pellucida is breached, using an acid solution or laser and a biopsy pipette is introduced through the hole into the embryo from where the cells are aspirated (De Vos and Streirteghem, 2001). This biopsy technique is used most often compared to others and allows detection of the maternal, paternal and postzygotic errors. It was earlier evaluated on mouse embryos by various groups before it was applied in human embryos (Nijs et al., 1988; Krzyminska et al., 1990; Kola and Wilton, 1991).

In blastocyst biopsy, the embryos are left to grow until the blastocyst stage which gives the advantage that more cells can be obtained for analysis. The cells that are biopsied are the trophectoderm cells and not the inner cell mass. Mosaicism of the trophoctoderm cells may increase the chances of misdiagnosis as later in pregnancy confined placental mosaicism (CPM) is observed (Kalousek and Dill, 1983). The main disadvantage of this biopsy method is that fewer embryos reach this stage in culture and the time left for diagnosis is limited. In a study performed by McArthur et al. (2005) 21% of the cases had no embryos suitable for biopsy.

## **1.5 Diagnosis techniques**

PCR and FISH are the techniques that are routinely used for clinical PGD and PGS cases. The method used depends on the disease. PCR is used when the couple is at risk of transmitting a single gene disease. In most cases the specific mutation has to be known (Piyamongkol et al., 2003). FISH is used to examine chromosomes in the case of Robertsonian or reciprocal translocations and for PGS.

### **1.5.1 Polymerase Chain Reaction**

PCR is one of the most powerful and useful tools of genetics. It was introduced 20 years ago by Kary Mullis who was awarded the Nobel Prize for Chemistry in 1993 (Mullis et al., 1990). PCR was the first method of analysis used in PGD and is still used for the identification of single gene diseases. Nowadays, more than 200 single gene diseases can be diagnosed in PGD with PCR and the number will increase as a result of increase in patient demands (Harper et al., 2008a). PCR for PGD requires more thermo-cycles to be performed than normal PCR because of the limited amount of DNA that is present (6-12pg). The limited amount of DNA is also responsible for certain problems, such as contamination, allele drop out (ADO) and failure of amplification, that make the diagnosis more complicated.

#### **1.5.1.1 Contamination**

Contamination is a risk of the PCR technique as DNA can become air-borne in the laboratory from cells or from previous PCR products and influence the result of the diagnosis especially in PGD, because there is minimal DNA available for diagnosis and stringent experimental conditions have to be taken to avoid contamination. The experimental conditions include performing the pre-PCR reaction in DNA free environments, separated from the post-PCR reaction, with separate laboratory coats, gloves, tubes, tips, pipettes, reagents and generally all the equipment that is used (Findlay

et al., 2001). In addition to the contamination that can occur from the laboratory, in PGD contamination can also occur from extraneous sperm that is embedded in the zona pellucida (paternal contamination) or cumulus cells that surround the embryo (maternal contamination) (Wilton et al., 2009). Paternal and maternal contamination can be avoided when specialized techniques are used such as intracytoplasmic sperm injection (ICSI) and removal of the cumulus cells (Marijo, 2000; Liebaers et al., 1998; Findlay et al., 2001). Further insurance that the cells examined are not contaminated from any other source is achieved by multiplex PCR which uses a set of highly polymorphic markers. These markers, usually short tandem repeats (STRs), are called “contamination markers” and have to be heterozygous and informative in the maternal and paternal DNA so that the embryonic cells should have one copy from each parent (Harper and Wells, 1999). The contamination markers used are usually closely located to the site of the mutation so that they can be linked. In case the phase of the mutation and the allele of the contaminated marker are known it would give even more reliable results. If more than two alleles of the contamination markers are present in the PCR product this would reveal that the samples were contaminated.

#### **1.5.1.2 Allele drop out**

Allele drop out (ADO) is the event where there is failure of the amplification of one of the two alleles in a cell heterozygous for this region. This produces an inaccurate result where the DNA can be considered as homozygous for that locus although it is not. ADO is unique to PCR where DNA is from one to five cells and it can affect either allele. There is no clear explanation of what causes ADO but assumptions suggest that it is a result of DNA degradation which leads to PCR-refractory breaks in both DNA strands (Piyamongkol et al., 2003). Furthermore, not optimised PCR conditions and incomplete cell lysis could lead to ADO because the DNA is not easily accessible (Thornhill et al., 2005). ADO only becomes a problem in PGD for dominant diseases and compound heterozygotes. In recessive mutations both alleles are the same and only one allele is expected but in dominant disorders there can be amplification of only the normal allele

which would lead to misdiagnosis (Wilton et al., 2009). ADO in optimised protocols for single cells can be as high as 20% (Piyamongkol et al., 2003).

Three different methods have been used in order to overcome ADO. The most important method is the use of highly sensitive fluorescent PCR (F-PCR) which is one thousand times more sensitive for the detection of PCR products than normal PCR. As F-PCR is more sensitive, ADO could be minimised in products that there is strong amplification bias in one of the alleles. In addition, linked markers, the same ones used for contamination, can be used in multiplex PCR together with the mutation primers in order to decrease the misdiagnosis which could appear because of ADO (Wells, 2004). This technique relies on the fact that ADO can be present irrespectively from the location of the primers even if the multiplex primers are very close. Again, it would be important to know the haplotype of the disease mutation and the linked marker. Even if one of the primers fails to show a result a conclusion can be drawn about the presence or not of the disease (Piyamongkol et al., 2003). For example, if ADO is 20% for the disease primers and the same for linked markers, and the phase is known, in multiplex PCR the overall ADO would be  $0.2 \times 0.2 = 0.04$  or 4%. PCR on DNA from two cells has shown to decrease the misdiagnosis resulting from ADO (Ao et al., 1998). In the case of three cells ADO is almost eliminated. This implies that ADO is a problem arising only in single cell diagnosis (Ao et al., 1998). However, when examining embryonic cells at cleavage stages a maximum of two cells can be obtained for diagnosis. Moreover, some embryos on day 3 are not able to lose two cells and only one cell is biopsied (Wells and Delhanty, 2001). Other techniques that would decrease ADO have been reported but so far there is still no reliable data of significant improvement. These techniques include increasing of denaturation temperature in the first ten cycles (Ray and Handyside, 1996), increasing the melting temperature and denaturation time (El- Hasmemite and Delhanty, 1997) and use of appropriate lysis buffers and polymerases (Thornhill et al., 2001).

ADO is still one of the greatest problems in PGD as all the methods mentioned above do not consistently eliminate it but only reduce it. ADO has been shown to have different rates in different types of cells. This is a fact that has to be taken in consideration because



in PGD research and work-up of PGD protocols different types of cells are analysed apart from blastomeres, such as buccal cells and lymphocytes (Piyamongkol et al., 2003).

#### **1.5.1.3 Total amplification failure**

Another problem that exists in single cell PCR is total amplification failure (TAF) which is the non-amplification of any of the alleles. Amplification failure may occur for the same reasons as ADO but it is also detected in homozygote alleles. TAF could also exist due to technical problems, like poorly optimised PCR protocols, incomplete cell lysis, anucleate cells or even human error where the cells are not transferred in the tube during the cell transfer (Wilton et al., 2009). In clinical PGD cases an embryo is excluded from transfer if the PCR of the blastomere shows TAF. However, this again results in a decreased rate of embryos that are available for transfer (Piyamongkol et al., 2003).

The problems that arise with PCR appear mainly due to the limiting amount of DNA present in a single cell that would not happen if the starting DNA was more. In order to overcome the problem of limited amount of DNA the entire genome of the cells can be amplified by whole genome amplification prior to molecular analysis.

#### **1.5.2 Whole genome amplification**

Whole genome amplification (WGA) is a relatively new tool in molecular biology that uniformly amplifies the entire genome with minimal bias and would have a substantial impact on the capability to perform comprehensive molecular and cytogenetic analysis using small patient specimens, even single cells (Luthra and Medeiros, 2004). WGA has been found to be very useful in different fields including cancer, forensic, paleo archaeological studies, PGD and most types of genetic tests where the DNA is limited (Luthra and Medeiros, 2004; Dean et al., 2002).

### 1.5.2.1 PCR based WGA techniques

Alu-PCR was the first reported WGA method (Nelson et al., 1989). The primers anneal to repetitive sequences such as Alu repeats which are distributed along the genome, and this leads to amplification of the entire genome and not just a small region (Nelson et al., 1989). This approach proved unsatisfactory as Alu repeats tend to be clustered in heterochromatic regions and the result was a bias amplification of these regions rather than the whole genome (Hughes et al., 2005). Nevertheless, this has led to the development of other PCR-based techniques with a similar philosophy, namely degenerate oligonucleotide primed PCR (DOP-PCR), primer extension preamplification (PEP), linker adaptor (LA) PCR, and GenomePlex.

The first reliable WGA techniques were DOP-PCR and PEP. DOP-PCR was first introduced by Telenius et al. (1992) and it has been used widely as an easy and reliable technique for whole genome amplification. The technique relies on standard PCR to amplify a specific region of the genome but with some modifications that allow the whole genome to be equally amplified. The differences are in the primers. In DOP-PCR only one set of primers is used that is partially degenerate (5'-CCGACTCGAGNNNNNNATGTGG-3'). This partial degeneration allows the primers to bind unspecifically to the DNA template at low stringency conditions. The second difference includes the thermal cycling condition, where in DOP-PCR there are two different stages. In the first five to eight cycles, there are low stringency conditions which allow the 3' end of the primer to bind unspecifically at sites of the genome and only according to the complementarity to the 6bp precise sequence of the 5' end. After these cycles, the conditions become more stringent by increasing the annealing temperature and with the facilitation of the random hexamer sequence (NNNNNN) it enables efficient primer annealing and amplification of only the initial fragments that are tagged with the specific part of the primer. This second round typically lasts for about 25-30 cycles (Telenius et al., 1992; Hughes et al., 2005). Several groups have tried to modify the technique by lowering the required amount of starting template, improving genome

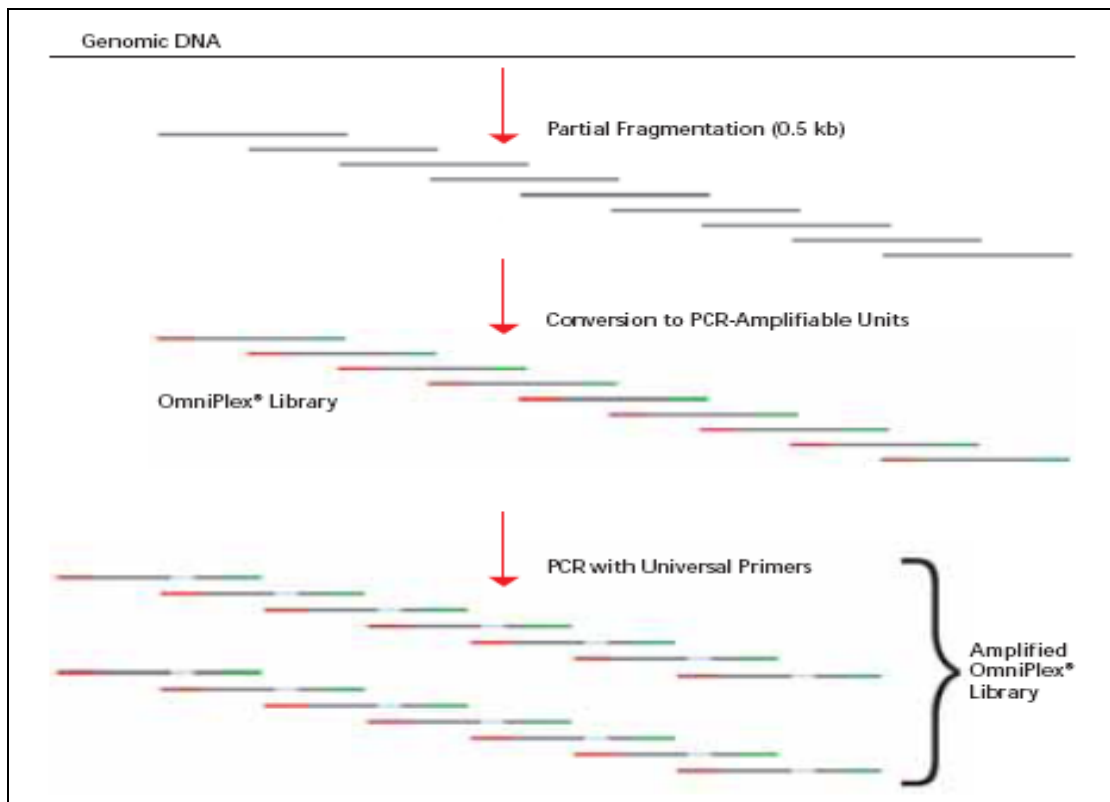
coverage and increasing the DNA yield, fidelity and fragment length (Kittler et al., 2002; Hirose et al., 2001; Larsen et al., 2001).

PEP was also developed in 1992 (Zang et al., 1992). The main difference with DOP-PCR is that PEP utilises complete degenerate primers of 15bp. In theory, the primer mix consists of  $10^9$  sequences ( $4^{15}$ ). PEP includes 50 PCR cycles in which the DNA is denatured at 92°C. The primers anneal in the beginning at 37°C for 2 minutes and then the annealing temperature increases by 0.1°C/second up to 55°C (Zhang et al., 1992). A more recent modification of this technique, which uses a polymerase with proofreading activity, further improves the technique (i-PEP) (Sun et al., 2005; Dietmaier et al., 1999).

The amount of DNA obtained after WGA with i-PEP is 0.2-0.8µg and 4-6µg after DOP-PCR. The product length ranges from 100-1000 bp for both techniques (Arneson et al., 2008; Bannai and Tokunaga, 2005; Sun et al., 2005). These techniques have been applied successfully in many applications including microsatellite analysis (Zheng et al., 2001), loss of heterozygosity (LOH) for cancer studies (Simpson et al., 2003), sequencing (Dietmaier et al., 1999), m-CGH (Harada et al., 2002), a-CGH (Peng et al., 2003), mutation detection by single strand conformation polymorphism (SSCP) and heteroduplex analysis (Bardaux et al., 2001). Successful amplification from degraded and damaged DNA has also been applied successfully (Heinmoller et al., 2002). PEP and DOP-PCR have been applied in clinical PGD and prenatal diagnosis (PND) (Peng et al., 2007).

GenomePlex is a relative new WGA technique based on PCR which seems to be more promising compared to the other PCR based WGA techniques (Little et al., 2005). Unlike DOP-PCR and PEP which use degenerate primers, this method is based on reformatting gDNA into “inherently-amplifiable DNA molecules of controllable size”, named plexisomes. In the GenomePlex reaction, the DNA is first randomly chemically fragmented to smaller pieces of average 500bp. Plexisomes are produced by attaching universal adapters to both ends of each fragmented DNA piece. The plexisomes, which form the OmniPlex library, are then amplified with the help of a single primer that binds

to the universal adapters and high-fidelity DNA polymerase via traditional PCR for limited cycles (Langmore, 2002; figure 1.1).



**Figure 1.1: Diagram of GenomePlex WGA method:** This figure illustrates the GenomePlex method for WGA. The genomic DNA is first fragmented into small pieces, converted to PCR amplifiable units (OmniPlex library) and then amplified with the use of universal primers that bind to the universal adapters. This figure was adapted from The Sigma-Aldrich official website ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)).

GenomePlex produces 500-1000 fold amplification of DNA with an average of 600bp product length (Arneson et al., 2008). The superiority of GenomePlex compared to other PCR-based techniques relies on the fact that there is no non-specific DNA formation and it can successfully amplify samples from degraded DNA (Little et al., 2006). Experiments have also shown that GenomePlex does not produce amplification bias (Barker et al., 2004). Although it has recently been developed, it has already been applied in different genetic tests, including high resolution analysis with a-CGH in samples from formalin-

fixed paraffin-embedded (FFPE) archival specimens (Little et al., 2006) and single cells (Fiegler et al., 2006). A clinical PGD application with GenomePlex was also reported recently (Chen et al., 2008).

#### **1.5.2.2 The Phi29 Polymerase**

Phi29 polymerase is the viral protein 2 found in bacteriophage Phi29 from *Bacillus subtilis* (Hermoso et al., 1985; Blanco et al., 1989). It is a protein-primed DNA-dependant replicase and belongs to the eukaryotic-type family of DNA polymerases (family B) (Blanco and Salas, 1996). This bacteriophage contains a linear, double-stranded DNA of 19,285 bp together with a terminal protein, p3, which is covalently connected to both 5'-ends of the DNA by a phosphodiester bond between the OH group of serine residue 232 and 5'- dAMP (Blanco et al., 1989). In vivo and in vitro studies have shown that Phi29 polymerase is the only enzyme that is required for the synthesis of the entire genome. The only other protein that is required for the synthesis of DNA is protein p3 which is used as the initiation primer. Moreover, genetic and biochemical analysis of the Phi29 polymerase have pointed out the necessity of this enzyme in DNA synthesis in vivo and in vitro (Inciale et al., 1980; Shih et al., 1982).

Phi29 polymerase consists of a single polypeptide of 66-kD (272 amino acids). Despite the small size for an enzyme it has multiple enzymatic activities in the single polypeptide chain which make it distinguishable from most other DNA polymerases (Rodriguez et al., 2005; Blanco and Salas, 1996). Its DNA polymerization activity includes high processivity and strand displacement during the polymerization process, which induces its catalytic properties. Phi29 polymerase has recently been found to owe these properties to a specific subdomain named terminal protein region 2 (TPR 2) and for this reason it has the highest processivity described for a DNA polymerase (>70 kb) (Rodriguez et al., 2005). In contrast to replication systems found in other organisms, such as *E. Coli*, where the presence of accessory proteins are crucial to confer processivity to the DNA polymerase by clamping the polymerase to the primer template, Phi29 polymerase does not need these proteins and is able to catalyze the reaction alone with high processivity.

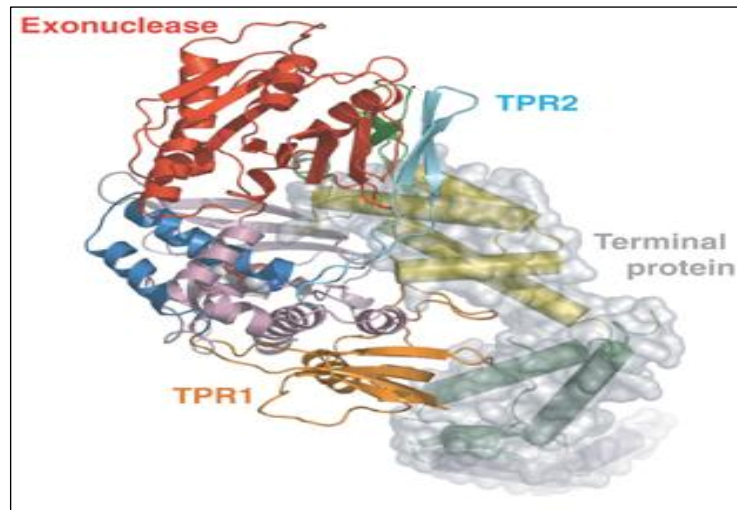
This probably arises from the unusually strong binding of the enzyme to single-stranded DNA (Blanco et al., 1989). The initial clamping is associated with the terminal protein p3 through deoxynucleotidylation and requires the presence of divalent metal ions (figure 1.2). Thus, the intrinsic insertion discrimination values range from  $10^4$  to  $10^6$  and with the help of 3'-5' exonuclease activity, this range is further improved 100- fold (Garmendia et al., 1992; Esteban et al., 1993).

Another interesting feature of Phi29 polymerase is that it has unwinding properties. Unwinding of the parental DNA helix is an important requirement for efficient replication of duplex DNA. In most DNA replication systems this is achieved by monomeric or multimeric enzymes which are called helicases and their role is to melt the dsDNA in an ATP-dependent fashion. In contrast to these systems, Phi29 polymerase is able to use double helical DNA templates without the help of any unwinding proteins. This again has been found to relative to the TPR 2 subdomain (Rodriguez et al., 2005).

Moreover, Phi29 polymerase initiates DNA replication at the origins which are located at both ends of the linear genome. This is done by catalyzing the addition of the initial dAMP onto the OH group of Ser-232 of the protein p3 which acts as a primer (Salas, 1991). Then, a transition stage occurs in which protein p3 priming changes to DNA priming. Finally, Phi29 polymerase replicates the entire genome progressively without dissociating from the DNA (Blanco et al., 1989; Rodriguez et al., 2005). The existence of two replication origins placed at both ends of the duplex DNA chromosome allows both strands to be replicated continuously (symmetric replication) in contrast to the classical discontinuous model which necessitates the synthesis of RNA-primed Okazaki fragments and additional accessory proteins (asymmetric replication). In this sense, the catalytic properties of the Phi29 polymerase, high processivity and strand displacement ability seem to be well designed to support this symmetric mode of replication (Maki et al., 1988; Blanco et al., 1989).

The unusual properties of the Phi29 polymerase, which have been studied for many years, have made it a revolutionary element for a completely different approach of WGA.

Specifically, the high processivity and the intrinsic strand displacement capacity of this polymerase have allowed the development of multiple displacement amplification (MDA) (Dean et al., 2002; Rodriguez et al., 2005).



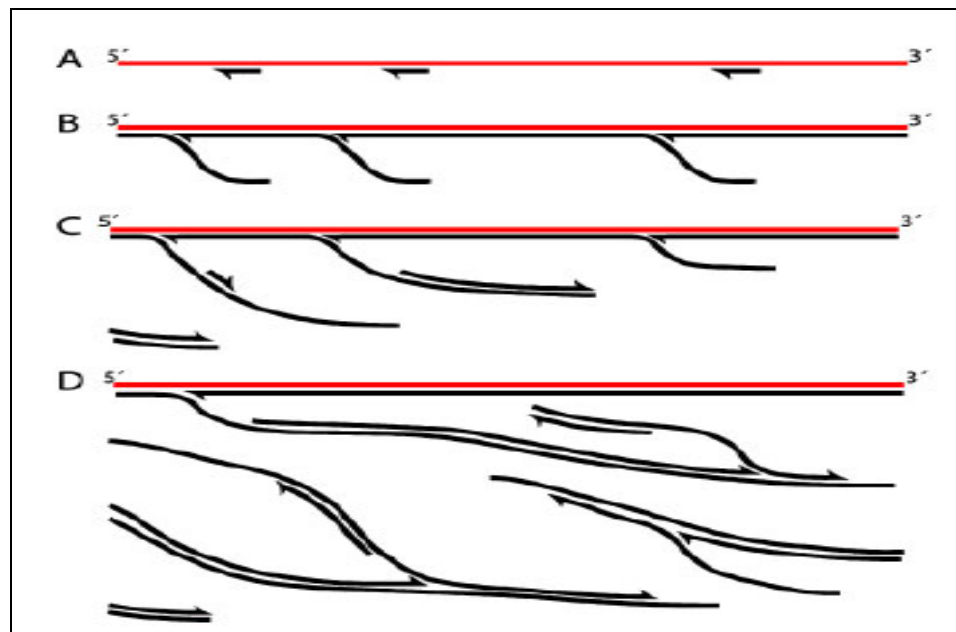
**Figure 1.5: Structure of Phi29 polymerase with terminal protein.** This figure shows the different parts of the Phi29 polymerase, including the TPR2 and the exonuclease activity region and the connection with the terminal protein p3. The figure was adapted from Kamtekar et al., 2006).

### 1.5.2.3 Multiple displacement amplification

MDA depends on previous findings of isothermal rolling circle amplification (RCA). RCA is a technique that uses Phi29 polymerase together with random hexamer primers to amplify large circular DNA templates such as plasmids and bacteriophage DNA (Dean et al., 2001; Dean et al., 2002). Interestingly, using the same reagents, linear genomic DNA could also be amplified. Initially a major problem with MDA reactions was the degradation of ssDNA due to the 3' to 5' exonuclease activity of the enzyme. This activity, in addition to the proof-reading capability, avidly degrades single-stranded oligonucleotides such as the random hexamer primers. In order to solve this problem, random hexamer primers that are protected from degradation were designed (exo-resistant) primers. These primers contain two phosphorothioate linkages at the

3'terminus. Thus, the primers are protected from degradation allowing amplification of genomic DNA (Dean et al., 2002).

For the reaction to take place, the genomic DNA has initially to be denatured. The denaturation is usually achieved by treatment with KOH. Then, the exo-resistant primers bind to the ssDNA. After that, Phi29 polymerase starts the extension of the primers. The exponential amplification is due to the ability of the enzyme to create a new replication fork whenever it meets dsDNA. Furthermore, Phi29 polymerase has the ability to invade the duplex 5' end of the downstream primer and create a new replication fork. More random primers bind to the displaced strand and the polymerase rapidly synthesizes more dsDNA. The overall result is thought to be a 'hyper-branched' structure which amplifies the entire genome in an exponential fashion (Lasken, 2005).



**Figure 1.6: Diagram of MDA.** MDA amplifies the DNA in an exponential fashion. First the hexamer primers bind to single stranded DNA (A), and then the polymerase starts to amplify towards the 5' end of DNA (B). Due to the helical activities, the downstream strand will unfold and a new single strand will appear (B). New primers will bind on the new single strand (C) and this will continue and amplify the DNA in an exponential fashion (D) (adapted from GE HealthCare Official Website, [www.gehealthcare.com](http://www.gehealthcare.com)).



As well as Phi29 polymerase, MDA can also be accomplished with the use of Bst polymerase and T4 gene 32 protein in conjunction with modified random primers instead of Phi29 polymerase. The difference between these two enzymes is that Phi29 polymerase has a greater strand displacement activity (SDA) than Bst polymerase/T4 gene 32 protein cocktail. However, Phi29 polymerase results in greater under and over representation of sequences when compared to the Bst enzyme (Lage et al., 2003).

WGA by MDA results in 80µg of DNA which ranges from 2kb up to 100kb product length with an average of 12kb. The amplification bias range between loci is less than 6-fold and the polymerase error rate is less than  $10^{-6}$ . MDA products from starting template as little as 0.03ng of gDNA could produce a number of robust molecular analysis tests (Dean et al., 2003) whereas Paez et al., (2004) estimated a 99.82% genome coverage of MDA products when the starting DNA material was 10ng. Although it is a recent development, MDA has already been applied to a-CGH (Lage et al., 2003), SNP analysis (Tranah et al., 2003), quantitative PCR (Dean et al., 2002) and microsatellite analysis (Hosono et al., 2003).

Very recently a new isothermal amplification technique was developed named primase-based WGA. This technique utilises the in vivo synthesis of primers rather than addition of synthetic primers by T7 gp4 primase and T7 polymerase for DNA replication at 37°C (Li et al., 2008). Nevertheless, this technique has not been validated yet.

### **1.5.3 WGA limitations**

Each WGA technique has advantages and disadvantages. The abilities of each WGA technique relies on the capacity of complete unbiased genome coverage, maximum fold amplification and yield, amplification product length, fidelity of the DNA polymerase and ability of amplification from small amounts of starting DNA template including single cells (Lasken and Egholm, 2003).

When the starting DNA template is more than 10ng and the DNA comes from fresh tissue, MDA seems to have much greater abilities compared to PCR based techniques. The large amount of DNA produced with high average length is in contrast with DOP-PCR, PEP and GenomePlex. Moreover, the amplification bias range between loci is  $10^3$ - $10^6$  fold in PEP and DOP-PCR compared to the 6- fold of MDA. Finally, the DNA polymerase error rate is less than  $10^{-6}$  whereas in PEP it is  $3 \times 10^{-4}$  to  $1 \times 10^{-5}$ , in DOP-PCR it is  $3 \times 10^{-4}$  and in i-PEP it is  $10^{-5}$  (Larsken and Egholm, 2003; Hughes et al., 2005). MDA can only give reliable results when the DNA comes from fresh tissue. PCR-based methods and especially GenomePlex have shown reliable results from poor-quality DNA (Little et al., 2006; Hughes et al., 2005).

One of the biggest challenges of WGA is to achieve robust amplification from less than 10ng of DNA as a starting template, including single cells where the DNA is only 6pg. All these techniques often produce a severe decrease of the fidelity of the experiment and increase of the ADO rate when the starting DNA template is less than 1ng (Kittler et al., 2002). Nevertheless, there are some groups that have managed successful results with less starting material (Huang et al., 2000). In 2005 the first clinical application of MDA for PGD was reported (Hellani, 2005).

WGA could be considered the key to overcome a major difficulty of PGD, which is the limited amount of DNA available for the diagnosis. So far, different WGA methods have been used in PGD for clinical purposes or for research in single cells, including blastomeres. MDA is the latest method of WGA but it is being used more often in PGD. MDA has been applied in clinical PGD in a number of cases including Marfan Syndrome (Lledo et al., 2006),  $\beta$ -thalassaemia and cystic fibrosis (Hellani et al., 2005), fragile X syndrome (Burlet et al., 2006) and for preimplantation genetic haplotyping (PGH) for Myotonic dystrophy and cystic fibrosis (Renwick et al., 2006). MDA was recently used for PGS using microarrays (Hellani et al., 2008). In research MDA has been successfully applied both for molecular analysis (Spits et al., 2006) but also for the use of a-CGH (Caignec et al., 2006) and m-CGH (Ng et al., 2005). DOP-PCR has limited use in clinical

PGD (Wells et al., 2002; Wilton et al., 2005) and research on single cells (Fragouli et al., 2006, Wells et al., 1998).

#### **1.5.4 Preimplantation genetic haplotyping**

Preimplantation genetic haplotyping (PGH) takes advantage of the amplification of the entire genome with WGA prior to molecular analysis. WGA of the DNA from a single blastomere is carried out prior to multiplex or singleplex PCR of many short tandem repeat (STR) markers for haplotyping. In order to reveal the haplotype of the embryo, the DNA from two affected relatives has to be obtained. As many tests have to be performed and only a single cell is used for the diagnosis, amplification of the entire genome with the use of whole genome amplification is necessary. The advantages of this technique is that it overcomes the difficulty of optimising the PGD case separately for each patient as it is a universal technique that can be applied in most patients and that the application of multiplex PCR is on large amounts of DNA rather than DNA from a single cell. Misdiagnosis could occur due to recombination events during meiosis (Renwick et al., 2007; Renwick et al., 2006).

#### **1.5.5 Fluorescent in situ hybridisation**

Fluorescent in situ hybridisation (FISH) is a method in which already produced fluorescently tagged DNA probes bind to specific complementary regions of the sample nucleus and form a stable DNA hybrid complex. In PGD the nuclei derive from blastomeres that are lysed onto a slide using lysis buffer and then are fixed. FISH probes are derived from cloned DNA fragments that have been inserted and amplified in vectors such as bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), plasmids and cosmids which are then fluorescently labelled (Knight and Flint, 2000). These probes can bind to repeat sequences (repeat sequence probes), to the telomeres of the short or long arms of the chromosomes (subtelomeric probes) or they can be locus specific and bind to specific regions of a chromosome corresponding to specific genes and their surrounding sequences (locus-specific probes). FISH is known to detect many

chromosomal abnormalities, it is very accurate and it is rapid as it can be applied directly to interphase nuclei (Haaf and Ward, 1994). As in PCR, it was first applied for sexing the embryos in order to overcome the problem of X-linked diseases but nowadays it is used for most chromosomal abnormalities. One disadvantage of interphase FISH is that the technique's efficiency decreases as the number of probes increases (Harper and Wells, 1999). Other problems that can lead to misdiagnosis are mosaicism and signal overlapping (Wells and Harper, 1999). A crucial point of FISH is the fixation of the embryonic nucleus on the slide which has to ensure no loss of genetic material and the best nuclear morphology. Apart from PGD, FISH is used in the vast majority of the PGS cases which involves the analysis of five to 15 chromosomes (Baart et al., 2007).

#### **1.5.6 Metaphase comparative genomic hybridisation**

M-CGH is a technique that allows the detection of chromosomal gains and losses of the entire genome of the cells that are being assessed (Weiss et al., 1999). It was first described by Kallioniemi et al (1992) and by Manoir et al. (1993) in order to identify chromosome abnormalities in tumours. In this technique the test DNA is labelled with green fluorescence and mixed with the same amount of a reference DNA, which is chromosomally normal, and is labelled with a red fluorescence. The mix is hybridised onto normal 46, XY metaphase spreads on a microscope slide. The test and reference DNA compete in order to bind to the complementary sites of the spread chromosomes. In case there is a chromosomal gain or loss in the test DNA, the proportion of green/red fluorescently labelled DNA at this specific site will not be equal to one and it would result in more green or red in this region. Computer software is used for analysis of each chromosome. In sites where the test and reference DNA are equal the result would be a yellow or orange colour. According to m-CGH experiments the crucial detection size is estimated to be about 10-20Mb but the resolution can be much higher according to the percentage of loss or gain of the chromosomes. For example, in case of no copies present at a region (100% deletion) the resolution can be up to 1-2MB (Lapierre and Tachdjian, 2005).

For research purposes single blastomere m-CGH was first applied 1999 (Wells et al., 1999; Voullaire et al., 1999). The first clinical PGS cases using m-CGH were reported in 2001 with single blastomeres and 2002 with polar bodies (Wilton et al., 2001; Wells et al., 2002). However, a crucial problem with m-CGH is that it requires five days for the experimental procedure and analysis and the embryos have to be cryopreserved which decreases implantation rates (Wilton, 2004). Polar body diagnosis does not require cryopreservation (Obradors et al., 2008). In addition, the amount of DNA available from a single cell is 6-12pg whereas a normal m-CGH experiment would require microgram quantities of DNA. This problem was solved with the use of WGA techniques.

### **1.5.7 Array comparative genomic hybridisation**

Microarrays are the most powerful tool in genetics as it allows the analysis of thousands of results simultaneously in a fast, reliable and sensitive way (Glentis et al., 2006a). The development started in the mid 1980s from two other techniques; enzyme-linked immunosorbent assay (ELISA) which was used for specific protein detection and dot blotting which was used for specific nucleic acid detection. The progress of microarrays has been very rapid, especially with the use of chemiluminescent and fluorescent substances instead of immunoassays, which are much more sensitive, and the use of an inert solid support, which is much more amenable to miniaturisation and fluorescence-based detection, so that the reaction could be restricted in microspots allocated in the solid support (Ekins and Chu, 1999). Microarrays were first developed for gene expression analysis but they were soon used for cytogenetic analysis. This type of microarray was named a-CGH and was first reported in 1997 (Solinas-Toldo et al., 1997). Since the human genome project has been completed the main interest in genetics is to understand how changes at the molecular and chromosomal level can lead to the complexity of the eukaryotic cellular network and how some changes may lead to genetic diseases and syndromes (Sevenet and Cussenot, 2003).

A-CGH is very similar and has the same principle as m-CGH. The basic difference is that the target DNA for hybridisation is not metaphase chromosomes but cloned DNA

segments, like BACs, PCR-generated sequences, cDNA clones or oligonucleotides. The use of cloned DNA segments allows automated and faster analysis of the results, higher resolution according to the number of target DNA spotted on the array and greater sensitivity due to the fluorescent dyes used (Shaffer and Bejjani, 2004). A-CGH has been very useful in various fields and especially in areas where undiagnosed and unknown cytogenetic malformations may occur, such as cancer studies, cases of unexplained mental retardation and spontaneous abortions (Schaeffer et al., 2004). The high resolution and the automation of the technique provide a clear advantage over karyotyping, FISH and m-CGH.

Apart from research purposes, a-CGH could be very helpful for clinical cytogenetic diagnosis and especially for prenatal diagnosis and PGS. However, the diagnosis could be difficult and tricky due to the huge amount of information provided by an array. As many regions of the genome include unknown polymorphisms and copy number variants (variant regions of at least 1,000bp in length) with unknown clinical outcome, a high resolution analysis of prenatal samples or single blastomeres make the interpretation of such experiments even more difficult (Shaffer and Bejjani, 2004).

As it is a very complex process, there is much time and effort required in order to design a microarray experiment so that it is biologically and statistically robust. Many different technologies and strategies for DNA microarrays have been developed for DNA probes, labelling, platforms, fabrication types, scanning, and data analysis and interpretation, each with advantages and disadvantages (Glentis et al., 2006a). In order to use the most appropriate microarray for the experiment, all the different technologies must be considered (Cheung et al., 1999).

#### **1.5.7.1 Probes**

The first step of a microarray experiment is to select the probes that will be immobilised to the platform. There are two basic types of probes for producing DNA microarrays; pre-synthesised DNA or *in situ* synthesised oligonucleotides (Cheung et al., 1999). Pre-

synthesised DNA probes can be PCR fragments or genomic fragments derived from library clones, such as BAC vectors and bacteriophage P1 vectors. The length of DNA clones varies from 200 bp up to several kilo-bases. Oligonucleotide probes are usually 20-80-mer long and are synthesised either on the microarray platform or by conventional synthesis before immobilisation to the platform (Choudhuri, 2004).

#### **1.5.7.2 Microarray Platforms**

One of the features of microarrays is the use of solid platforms on which probes are immobilised. Solid platforms can be made from silicon-based materials or synthetic polymers, such as nylon, nitrocellulose and glass (Cai et al., 2002). Research on different types of surfaces to which DNA can bind has led to the conclusion that solid, impermeable surfaces have a variety of advantages over porous membranes and gel pads (McGall, 1997). The solid membranes are non-porous and the liquid cannot penetrate the surface, which gives direct access to the targets to hybridise to the probes without diffusing in the pores. Good mixing is required to enhance the rate of hybridisation (Duggan et al., 1999). The washing step is much easier, faster and improves reproducibility because there is no diffusion in surfaces made from rigid substrates. In addition, characteristics like flatness, rigidity and transparency are advantageous for image acquisition and processing. Finally, solid platforms benefit the incorporation into flow cells for the automated process which is essential for high throughput analysis (Duggan et al., 1999).

Glass surfaces also have unique advantages over nylon and other solid surfaces used to make microarrays. DNA can attach both covalently and non-covalently on glass surfaces according to how they have been treated (Baum et al., 2003). Glass surfaces are more tolerant to high temperature and washes with high ionic strength compared to other surfaces. Glass helps minimize the background noise-to-signal ratio because it has low fluorescence. Two different targets can be labelled with different fluorochromes and simultaneously incubated to the microarray in a single reaction, something that it is not possible with nylon surfaces (Baum et al., 2003).

### 1.5.7.3 Fabrication

There are two main methods for immobilising the probes on the microarray platforms; the *in situ* synthesis and the deposition by spotting. The photolithographic technique or chemical synthesis is used for *in situ* synthesis of the oligonucleotides (Choudhuri, 2004). The photolithographic method uses synthetic linkers that are modified with photochemically removable protecting groups and photolithographic masks to direct the light in the desired areas (Lipshutz et al., 1999). New photolithographic methods have been developed that use a digital micromirror device (DMD) and digital light processor (DLP) with the advantage being that high-density arrays can be produced in a cheaper way (Hardiman, 2004).

In spotting arrays, the probes are usually chosen from databases (GenBank, dbEST and UniGene) (Bowtell, 1999). The DNA has to be purified so that there is no contamination to the microarray platform. In PCR the DNA is usually purified by precipitation and gel-filtration in order to remove salts, detergents, PCR primers, proteins and cellular components that are usually found in the PCR mixture (Duggan et al., 1999). Robotic technology is then used in order to collect and deposit the purified DNA. The DNA transfer uses pins or needles (Choudhuri, 2004). The printing is achieved by contact of the pin with the surface that delivers the solution. The pins are moved to the next spot and the cycle is repeated. A few thousand to 30,000 probes can be printed on the microarray surface. The performance of the high-speed robotics mainly relies on the shape, reproducibility and durability of the pins (Bowtell, 1999). Uneven pins may deliver unequal DNA solution and influence the analysis of the experiment. Ink-jetting is a more advanced technique that has been developed which sprays the DNA on the surface. The advantage is there is no contact between the pin and the solid surface and so ink-jetting ensures that the spots have a more uniform size (Choudhuri, 2004).



#### **1.5.7.4 DNA sample labelling**

The next step of a microarray experiment is to label the sample DNA that will hybridise with the probes. One crucial factor is the purification of the targets prior to labelling because cellular components, such as proteins, lipids and carbohydrates can mediate significant non-specific binding of the labelled targets to the solid surface (Duggan et al., 1999). Two-colour experiments are conducted for a-CGH where two different labelled samples (target and control) are co-hybridised in a competitive way and results are defined in ratiometric terms (Hardiman, 2004). Radioactive elements, such as  $^{35}\text{P}$  dCTP, are not ideal for microarray experiments because of the microarrays physiology. Microarray elements are physically close to each other and strong hybridisation with a radioactive target may cause problems as it can easily interfere with detection of weak hybridisation in surrounding targets. Fluorescent labels (cyanine Cy3 and Cy5 and Alexa fluor 555 and 647 dyes) have many advantages. They have good photostability and yield and they are widely separated in their excitation and emission spectra allowing highly discriminating optical filtration (Duggan et al., 1999).

Labelling can be achieved with direct or indirect incorporation of the fluorochromes in the DNA samples. In direct incorporation, fluorochromes incorporate to the DNA sample directly with the use of Klenow fragment enzyme whereas in indirect incorporation there is a two step reaction. First amino-allyl deoxyridine triphosphate (AA-dUTP) is incorporated in the nucleic acid and then the fluorochromes bind to the nucleic acid by chemical coupling with AA-dUTP.

### **1.6 Prenatal diagnosis and screening**

Prenatal testing can be separated in screening and diagnosis. Screening tests involve ultrasonography for the visualisation and examination of the fetus by appraising different measurements, such as nuchal translucency, and maternal serum screening which measures the levels of specific proteins, such as pregnancy-associated plasma protein A (PAPP-A),  $\alpha$ -feto-protein, inhibin A,  $\beta$ -human chorionic gonadotrophin (hCG), hyper-

glycosated hCG and oestiol in the maternal blood. Screening is not 100% accurate. Invasive diagnosis methods are performed in pregnant women who have a history of previous miscarriages, have a genetic condition in the family, or when ultrasound and maternal serum screening have shown indications for congenital malformation of the fetus. The diagnostic tests include amniocentesis and chorionic villus sampling (CVS) in which fetal DNA is obtained for genetic diagnosis from which an accurate result can be obtained. Amniotic fluid is collected by amniocentesis between the 14<sup>th</sup> and the 20<sup>th</sup> week of gestation whereas chorionic villi are collected by CVS from 9.5 until 12.5 weeks. Fetal DNA is extracted for molecular or molecular cytogenetic analysis or fetal cells are cultured and prepared for cytogenetic analysis of chromosomal abnormalities by karyotyping.

Another alternative invasive technique for prenatal diagnosis is coelocentesis in which coelomic fluid is aspirated in order to obtain fetal cells (Jurkovic et al., 1993). Coelomic fluid is present inside the exo-coelomic cavity which is developed in pregnancies in the 4<sup>th</sup> week (Boyd and Hamilton, 1967). The exo-coelomic cavity is formed by a membrane that separates it from the amniotic cavity and is clearly identified with the use of high resolution trans-vaginal ultrasound (Jauniaux et al., 2003). Coelomic fluid can be aspirated from the 5<sup>th</sup> until the 12<sup>th</sup> week of gestation with the use of a needle that is trans-vaginal inserted into the exo-coelomic cavity under ultrasound guidance. The great advantage of this technique is that prenatal diagnosis can be done much earlier compared to the other techniques. However, the limited amount of DNA is a major drawback for clinical application and it is currently not performed for prenatal diagnosis.

## **1.7 Aims**

Whole genome amplification can be considered a powerful tool in molecular biology, especially in the fields where DNA is limited and precious. Very good results are achieved when the starting DNA template is more than 1ng and is good quality. However, in many genetic fields good quality DNA and such quantities cannot be obtained. In PGD, where the DNA derives from a single cell only, WGA could be the key for overcoming the majority of the problems that are encountered in single cell

diagnostics. The main aim of this project was to produce WGA products from single cells or small numbers of cells in order to achieve robust and reliable molecular and molecular cytogenetic analysis. This project concentrated on the advantages and disadvantages of different techniques to determine when and how WGA could be useful when there are small quantities of DNA. Although the main focus was on PGD, single cell analysis could be very useful in different areas, including blastomere research on aneuploid mechanisms, prenatal diagnosis, cancer research and forensic sciences.

The project was separated into three chapters. In the first chapter, single cell WGA was evaluated for molecular analysis with the use of PCR. PCR is one of the fundamental tools of molecular biology and it is widely used in clinical PGD for monogenic diseases. However, at the single cell level, optimisation of a PCR protocol is difficult and time consuming, whereas more DNA would be beneficial for performing multiple experiments, haplotyping and repeating an experiment that has failed. Hypothesis: Can MDA be used as a universal step prior to molecular analysis for PGD?

The aim of the molecular project was to assess the efficiency and the accuracy of MDA on single cells using well established PCR protocols that have been optimised and applied in clinical PGD. The reason for choosing optimised PCR protocols was to eliminate the chances that experiments would not work due to PCR insufficiency. Different cell types (lymphocytes, fibroblasts, buccal cells and blastomeres) were considered and compared. Each cell type may respond differently due to different DNA quality and compaction. Although blastomeres are the key cell types for PGD, lymphocytes and buccal cells are widely used for optimisation protocols prior to a clinical case. Cell types that respond in a similar way to blastomeres should be used for optimisation of clinical PGD cases. Three different lysis methods were compared for the lysis of the cells prior to WGA as lysis plays a crucial role in the way the DNA becomes accessible to the WGA enzymes. These were an enzymatic lysis (proteinase K), an osmotic lysis (alkaline lysis buffer, ALB) and a modification of the ALB lysis where DTT was excluded from the lysis buffer. DTT was excluded in the ALB modified method because it has been may damage the DNA (Spits et al., 2006). For the molecular

project the only WGA method used was MDA because it is the only method that produces long DNA strands which are necessary for molecular analysis when the DNA comes from a single cell.

In the second chapter two different WGA techniques were used to amplify DNA from single cells and then the products were analysed by a-CGH. Hypothesis: Is it possible to accurately assess the chromosomes of a single cell by a-CGH?

The WGA techniques used were MDA and GenomePlex, which give the best genomic representation and the minimum amplification bias. Lymphocytes, fibroblasts and blastomeres were used as sources of single cells to obtain DNA. Buccal cells were excluded from this project due to their poor quality of DNA. Proteinase K and ALB without DTT were used for single cell lysis. BAC a-CGH slides of different resolutions (10MB-100KB) were used. The aims of this project were to test if WGA products from single cells can be applied in a-CGH and if a high resolution could be achieved in order to detect small chromosome abnormalities. The motivation for this project was that microarrays are considered to be a very powerful tool in molecular biology and they were tested to determine if they would work at the single cell level. The benefits of optimising single cell a-CGH would not only be reflective in clinical PGD and PGS but it could also invade research areas related to single cells, including tumour cells, where chromosomal analysis of the entire genome of specific cells would be achieved. Although WGA products from single cells have been successfully applied in m-CGH, the use of a-CGH could increase the resolution analysis, minimise the time for diagnosis and will automate the technique.

In the final chapter, DNA extracted from coelomic fluid was amplified by MDA and GenomePlex and analysed by a-CGH. Hypothesis: Can aneuploidy be detected in coelomic fluid using a-CGH?

The aim of this project was to assess if DNA extracted from coelomic fluid could be successfully analysed by a-CGH. The challenge of this project was that the poor quality

and very small amount of DNA present in coelomic fluid may hamper the analysis by a-CGH. Because minimum collection of coelomic fluid has been suggested to limit fetal loss by coelocentesis, the maximum volume of coelomic fluid used for WGA was 1ml whereas in two cases WGA was applied directly on 5 $\mu$ l of coelomic fluid without DNA extraction. Although FISH and PCR experiments have been successfully performed with coelomic fluid samples, a-CGH had not been previously achieved. A limited factor for not performing coelocentesis for early prenatal diagnosis is the poor quality and quantity of DNA obtained. However, overcoming this problem with WGA and managing successful application of a-CGH on coelomic fluid samples would enhance the use of coelocentesis as a new technique of early prenatal diagnosis.

## **Chapter 2**

### **Materials and methods**

#### **2.1 General**

Materials and methods that are specific for each chapter are written within each chapter separately. In this chapter all the materials and methods that were used in all or most chapters are described, including DNA extraction, single cell lysis and isolation and whole genome amplification.

##### **2.2.1 DNA extraction from blood**

Generally, good quality DNA for positive controls and STR allele sizes was obtained from whole blood after a standard DNA extraction protocol. The materials related with DNA extraction are listed in appendix A2. For DNA extraction, 5ml of blood collected in ethylenediaminetetraacetic acid (EDTA) tubes were mixed with the same amount of low salt buffer TKM1 in a 14ml centrifuge tube. Then, 125µl of the detergent Igepal (Sigma, UK) was added in order to lyse the cells and the solution was mixed well and centrifuged at 2,200 rpm for 10 minutes. The supernatant was discarded and the remaining pellet was re-suspended in 5ml of TKM1 and 125µl of Igepal, mixed and centrifuged at 2,200 rpm for 10 minutes. This was repeated a number of times until the redness of the pellet was reduced. The pellet was re-suspended in 100µl of TKM1 and the sample was transferred to a 1.5ml microfuge tube (Eppendorf, UK). In the solution, 0.8ml of TKM2 and 50µl of 10% sodium dodecyl sulphate (SDS; Sigma, UK) were added and mixed well in order to lyse the white blood cells. The sample was incubated at 55°C for 30 minutes or longer until the lumps had gone. Then 300µl of 6M NaCl were added, mixed well and centrifuged at 12,000 for 5 min. The supernatant was transferred into two new 1.5ml microfuge tubes and the pellet was discarded. The DNA was precipitated by adding 1ml of 100% ice cold ethanol and mixing gently. The sample was centrifuged at 13,000 rpm for 10 minutes, the supernatant was discarded and the pellet was re-suspended in 1ml of ice-cold 70% ethanol. The sample was centrifuged for 5 minutes at 10,000 rpm, the

supernatant was discarded and the pellet was left to air dry for 5 minutes. Finally, 50µl of TE buffer (10mM Tris; **1mM** EDTA pH 8.0) was added to the sample.

### **2.2.2 Culturing, collection and DNA extraction from skin fibroblasts**

DNA extracted from fibroblasts was used for molecular and cytogenetic experiments. Two trisomic fetal skin fibroblast cultures were provided from frozen cell stocks that grew in monolayer cultures at 37°C. For cell collection, the cell culture medium was poured off and 5ml of prewarmed (37°C) Hank's medium was added to wash the cells. After a few seconds, the solution was substituted with 5ml of 0.25% trypsin/Versene solution at 37°C and the flask was incubated at 37°C for 5 minutes. During this period most of the cells were detached from the flasks and could be seen under an inverted microscope. A few drops of fetal calf serum were added in order to inactivate the trypsin and the cell suspension was placed into centrifuge tubes and spun for 5 minutes at 1000rpm. Following centrifugation, most of the supernatant was discarded and 2ml of PBS was added in order to resuspend the pellet. The cell suspension could be used for DNA extraction, single cell isolation and slide preparation for FISH.

For DNA extraction the cells were lysed by the addition of 2.5ml proteinase K solution and incubated at 37°C for 30 minutes. Equal volumes of isopropanol were added to the tubes in order to precipitate the DNA and with the use of sterile inoculation loops the DNA was transferred into microcentrifuge tubes. The DNA was centrifuged for 5 minutes at 10,000 rpm, the supernatant was discarded and the pellet was left to dry at room temperature for 5 minutes. The DNA was finally suspended in 200µl of TE buffer and stored at 4°C.

### **2.2.3 Lymphocyte and buccal cells collection**

Lymphocytes and buccal cells that were used for the projects had to be collected prior to single cell isolation and cell lysis. All the materials regarding cell collection, single cell isolation and cell lysis are listed in appendix A4. For lymphocytes, suspensions were

obtained from normal individuals after fresh whole blood collection in lithium heparin tubes. Six ml of the collected blood was mixed with 6ml of saline (0.9% NaCl) solution in a 14ml centrifuge tube (blood/saline: 1/1). The mixture was transferred into another 14ml centrifuge tube that contained Ficoll-Paque plus (Sigma, UK). The blood/saline solution was dropped gently in a way that it formed a layer on top of the Ficoll-Paque plus without mixing it. The amount of blood/saline solution/Ficoll-Paque plus was 4/3. The sample was centrifuged at 1300 rpm for 30 minutes and the centrifuge stopped slowly without the use of the brake. After centrifugation the lymphocytes formed a buffy coat in the middle of the tube and were transferred into a new 14ml tube. The tube was filled up with saline solution and the solution was mixed. The solution was centrifuged for another 15 min at 1300 rpm. The supernatant was discarded and the remaining pellet was dissolved in 2ml of saline solution. After dissolving the pellet the tube was filled up with saline and the solution was centrifuged again for 15 min at 1300 rpm. The supernatant was discarded and the remaining pellet was dissolved in 2ml of saline solution. The cells were stored on ice for single cell isolation or for FISH experiments for a maximum of 16 hours.

Buccal cells were collected from the buccal cavity by rubbing it with a sterile swab and transferred in a sterile 1.5ml tube with 1ml of phosphate buffer saline (PBS, Sigma, UK) and polyvinyl alcohol (PVA, Sigma, UK) (1g of PVA in 50ml of PBS) (Gibco, UK). The cells were stored for up to one day at 4°C.

### **2.3 Single cell isolation and cell lysis**

The single cell isolation protocol was different according to the type of cell lysis that was used. There were two single cell isolation protocols; alkaline (ALB lysis) and proteinase K (PK lysis).

In order to isolate single cells, a small drop (~10µl) of the cell suspension was placed into a plastic Petri dish. For alkaline lysis, drops of dissociation buffer (DB; 140mM NaCl, 0.2mM KCl, 0.04mM NaH<sub>2</sub>PO<sub>4</sub> X 2H<sub>2</sub>O, 5.5mM glucose, 1.2mM NaHCO<sub>3</sub>, 0.02mM



EDTA, and 0.01% (w/v) phenol red) (Sigma, UK) containing 1 mg/ml bovine serum albumin (BSA, Sigma, UK) were dropped onto another plastic Petri dish. Single cells were picked up under an inverted microscope by a plastic capillary using aspiratory micromanipulation and they were washed three times in the DB drops. The isolated cells were transferred by aspiration to a 0.5ml tube which contained 2.5µl of alkaline lysis buffer. The alkaline lysis buffer consisted of 200mM KOH (lysis 1, L1) or 200mM KOH and 50mM dithiothreitol (DTT, Sigma, UK) (lysis 2, L2) (table 2.1). The cells were incubated at -80°C for at least 30 minutes and the cell lysis was completed by incubating the tubes that contained the cells at 65°C for 10 minutes.

For PK lysis, drops of PBS/PVA were dropped onto the plastic Petri dish. Similar to the alkaline lysis, single cells were picked up under the microscope by a plastic capillary using aspiratory micromanipulation and after washing three times in the PBS/PVA drops to ensure the single cell isolation, they were transferred by aspiration to a 0.5ml tube which contained 1µl of cold 17µM SDS (Sigma, UK) and 2µl of 125µg/ml proteinase K (Roche Diagnostics, USA) (lysis 3, L3) (table 2.1). The lysis was completed by incubating the mixture at 37°C for 1 hour and then inactivating the enzyme at 95°C for 15 minutes in a PCR machine (Eppendorf, UK). The lysed cells were stored at 4°C for immediate use or stored at -80°C for use within a week (Table 2.1).

A negative control sample was collected after each cell was isolated by touching the capillary to the last drop that contained the isolated cell and transferring the volume to a new tube containing one of the lysis buffers. The negative samples were stored at -80°C and used directly for single cell PCR for the DM triplex to ensure absence of contamination.

Lysis method	Isolation medium	Type of lysis	Characteristics
L1	Dissociation buffer with BSA	Alkaline (200mM KOH)	Osmotic (burst the cell) milder without DTT in buffer
L2	Dissociation buffer with BSA	Alkaline (200mM KOH with 50mM DTT)	Osmotic (burst the cell) stronger with DTT in buffer
L3	PBS/PVA	Enzymatic (proteinase K)	Enzymatic lysis (digestion of proteins and free the DNA)

**Table 2.1: Summary of different lysis methods used for single cell lysis.** This table summarises the three different lysis methods used; alkaline lysis without DTT, alkaline lysis with DTT and enzymatic lysis with the use of proteinase K.

## 2.4 Whole genome amplification

Two different methods of whole genome amplification, the isothermal method of multiple displacement amplification (MDA, Qiagen, UK) and the PCR-based method using fragmentation of DNA and universal primers (GenomePlex, Sigma, UK), were applied on genomic DNA and single cells before using these products for molecular or molecular cytogenetic analysis. WGA both for MDA and for GenomePlex was accomplished with the use of kits from companies. All the reagents and materials used for MDA and GenomePlex reactions are listed on appendix A5. A total of seven protocols were produced (four protocols for MDA products and three protocols for the GenomePlex reactions) for the amplification of single cells and gDNA (table 2.2).

### 2.4.1 MDA

MDA amplification was achieved using the Repli-g Midi kit (Qiagen, UK). For single cells, the MDA reaction took place after cell lysis from one of the previous mentioned lysis methods (L1, L2 and L3, section 2.3). The protocol followed was the one proposed by the company for gDNA. Briefly, 2.5µl of denaturation buffer (D1; 15% reconstituted DLB buffer) was added to the ~3µl of lysed product and incubated at room temperature for 3 minutes. Then 5µl of neutralisation buffer (N1; 10% of Stop solution) was added to mixture. A master mix of 10µl dH<sub>2</sub>O, 29µl Repli-g buffer and 1µl Repli-g DNA

Protocol name	DNA source	Lysis Method	WGA technique	Denaturation of DNA	Neutralisation	Amplification method	Modifications
P1	Single cell	L1, L2 or L3	MDA	2.5µl D1	5µl N1	30°C for 6 hours	MDA company original protocol for gDNA
P2	Single cell	L1	MDA	2.5µl D1	5µl N1	30°C for 2 hours	2 hours incubation instead of 6 hours
P3	gDNA	-	MDA	5µl D1	10µl N1	30°C for 2 hours	2 hours incubation instead of 6 hours
P4	Coelomic fluid	L2 (from WGA kit)	MDA	3.5µl D2	3.5µl Stop solution	30°C for 2 hours	2 hours incubation, MDA company protocol for direct application on tissues
P5	gDNA	-	GenomePlex WGA2	95°C for 4 minutes	-	14 PCR cycles	WGA2 company original protocol
P6	Single cell	L3 (from WGA kit)	GenomePlex WGA4	99°C for 4 minutes	-	25 PCR cycles	WGA4 company original protocol
P7	Single cell	L1	GenomePlex WGA4	99°C for 4 minutes	5µl N1	25 PCR cycles	WGA4 ALB L1 lysis, no incubation with PK enzyme

**Table 2.2: Protocols of DNA source and modifications of the WGA techniques.** This table summarises all the different protocols of WGA applied for the molecular and a-CGH projects. Protocol P1 was used for the molecular project, protocols P2, P3, P6 and P7 were used for the single cell a-CGH project and protocols P3, P4 and P5 were used for the coelomic fluid a-CGH project.

D1: denaturation buffer 1

D2: denaturation buffer 2

N1: Neutralisation buffer 1

polymerase for each sample was prepared and 40µl of the master mix was added to each sample. Finally the tubes were incubated at 30°C for 6 hours (protocol 1, P1) or 2 hours (protocol 2, P2). The reaction was terminated by heating the samples at 65°C for 3 minutes and storing them at 4°C.

For gDNA the initial DNA template used was 5µl. The protocol used (protocol 3, P3) was very similar to P2 with the only differences being double amount of D1 (5µl instead of 2.5µl) and N1 (10µl instead of 5µl) added to the DNA template and difference in the master mix (30µl including 29µl Repli-g buffer and 1µl Repli-g DNA polymerase instead of 40µl master mix which had an additional 10µl of dH<sub>2</sub>O). The incubation time for gDNA was 2 hours at 30°C.

For the prenatal chapter (chapter 5) direct amplification of 5µl coelomic fluid without previous DNA extraction was accomplished (protocol 4, P4). Briefly, 5µl of coelomic fluid was mixed with 3.5µl of denaturation buffer D2 (5µl of 1M DTT, 55µl of reconstituted buffer DLB) and incubated for 10 minutes on ice followed by 3.5µl of Stop solution. A master mix of 8µl dH<sub>2</sub>O, 29µl Repli-g buffer and 1µl Repli-g DNA polymerase for each sample was prepared and 38µl of the master mix was added to each sample. The tubes were incubated at 30°C for 2 hours (protocol 4, P4) and the reaction was terminated by heating the samples at 65°C for 3 minutes before storing them at 4°C.

#### **2.4.2 GenomePlex**

Two kits were used for amplification of DNA; the WGA2 kit (WGA2; Sigma, UK) which was used for the amplification of gDNA and the WGA4 kit (WGA4; Sigma, UK) which was used for the amplification of single cells.

With the WGA2 kit, 1µl of 10x DNA fragmentation buffer was added to 10µl gDNA in a 0.5ml PCR tube. The mixture was first heated exactly for 4 minutes at 95°C and then cooled on ice immediately. After brief vortexing and centrifugation to consolidate the products, 2µl and 1µl of 1x library preparation buffer and library stabilisation solution

respectively were added to the sample. The mixture was heated at 95°C for 2 minutes and cooled on ice. For OmniPlex library generation, 1µl of library preparation enzyme was added, the samples were vortexed and centrifuged briefly and incubated in a thermal cycler at 16°C for 20 minutes, then at 24°C for 20 minutes and finally at 37°C for 20 minutes. The reaction was terminated by heating the samples at 75°C for 5 minutes and storing at 4°C. For the PCR amplification, a master mix consisting of 7.5µl of 10x amplification master mixes, 47.5µl of nuclease-free H<sub>2</sub>O and 5µl of WGA DNA polymerase per reaction was formed and 60µl of this master mix was added to each sample. The PCR amplification consisted of an initial denaturation step of 95°C for 3 minutes followed by 14 cycles with a denaturation step of 94°C for 15 seconds and an anneal/extension step of 65°C for 5 minutes. The samples were stored at 4°C or -20°C (protocol 5, P5).

For single cells the GenomePlex Single Cell WGA Kit (WGA4) was used. The protocol used was very similar to P5 with the difference that an enzymatic lysis method was also included prior to DNA fragmentation (protocol 6, P6). For the cell lysis, 8µl of nuclease free H<sub>2</sub>O were added to the tube which contained the single cell in PBS/PVA solution in order for the volume to reach 9µl. A fresh lysis and fragmentation buffer solution were prepared (2µl of proteinase K solution and 32µl of 10x single cell lysis and fragmentation buffer) and 1µl was added to the tube. The mixture was vortexed and centrifuged briefly prior to incubation at 50°C for 1 hour and then heated at 99°C for four minutes. After that stage the protocol was identical to P5 with the exception of the final thermocycling step in which the products were first denatured at 95°C for 3 minutes and then 25 cycles were performed that had a denaturation step of 94°C for 30 seconds followed by an annealing/extension step of 65°C for 5 minutes.

A modified protocol for the GenomePlex Single Cell WGA Kit was also introduced in which the cell was lysed by the L1 method (protocol 7, P7). In this case the proteinase K solution was substituted by nuclease free water and the samples were not incubated at 50°C for 1 hour. A summary of the different protocols for WGA are listed on table 2.2.

## **Chapter 3**

### **Molecular analysis of MDA products from single cells**

#### **3.1 Hypothesis**

The hypothesis is that MDA can be used as a universal step prior to molecular analysis for PGD.

In order to address this hypothesis, the efficiency and accuracy of MDA as a method of WGA was measured on different cell types (buccal cells, lymphocytes, fibroblasts and blastomeres) and lysis methods (osmotic lysis L1 and L2 and enzymatic lysis L3) using two multiplex PCR protocols (DM I and CF) that had been optimised and applied in clinical PGD. The findings would be beneficial both for research and clinical PGD and could also be useful in other fields where the DNA is limited. The advantages and disadvantages of the use of MDA on single cells can be identified in order to obtain the best use of this technique in PGD.

#### **3.2 Introduction**

MDA seems to give the best results when the initial DNA template is from good quality gDNA compared to most PCR-based techniques (Lasken et al., 2005). MDA superiority is due to greater genome coverage, less amplification bias and lower polymerase error rate. MDA's big advantage over GenomePlex is the high molecular weight products that are produced. Thus, GenomePlex could not be used for single cell amplification prior to PCR due to the low molecular products that produces. So far studies with MDA on DNA sources of limited amount include microbial genomes (Lasken, 2007), bovine blastomeres (Hirayama et al., 2006), blood spots (Dean et al., 2002), epidemiologic studies on buccal cytobrush (Moore et al., 2007) and forensic studies (Barber and Foran, 2006). MDA application on single cells for PGD have been reported both for clinical and research purposes (Ren et al., 2007; Renwick et al., 2006; Lledo et al., 2006; Burlet et al.,

2006; Hellani et al., 2005). The main advantage of MDA in single cells is the final volume of DNA which is created and it can be used for PGH (Renwick et al., 2006), HLA typing (Hellani et al., 2005) and for multiple tests to minimise the chance of misdiagnosis (Obradors et al., 2008). Although all the WGA methods produce large amounts of DNA, the PCR-based WGA methods cannot be used in molecular analysis due to the short DNA products that are produced. This is because in single cell PCR there are only two copies of the genome and there is a high risk of the DNA being cut in the area that would be the target for PCR amplification. So in this chapter only MDA was used as a WGA method.

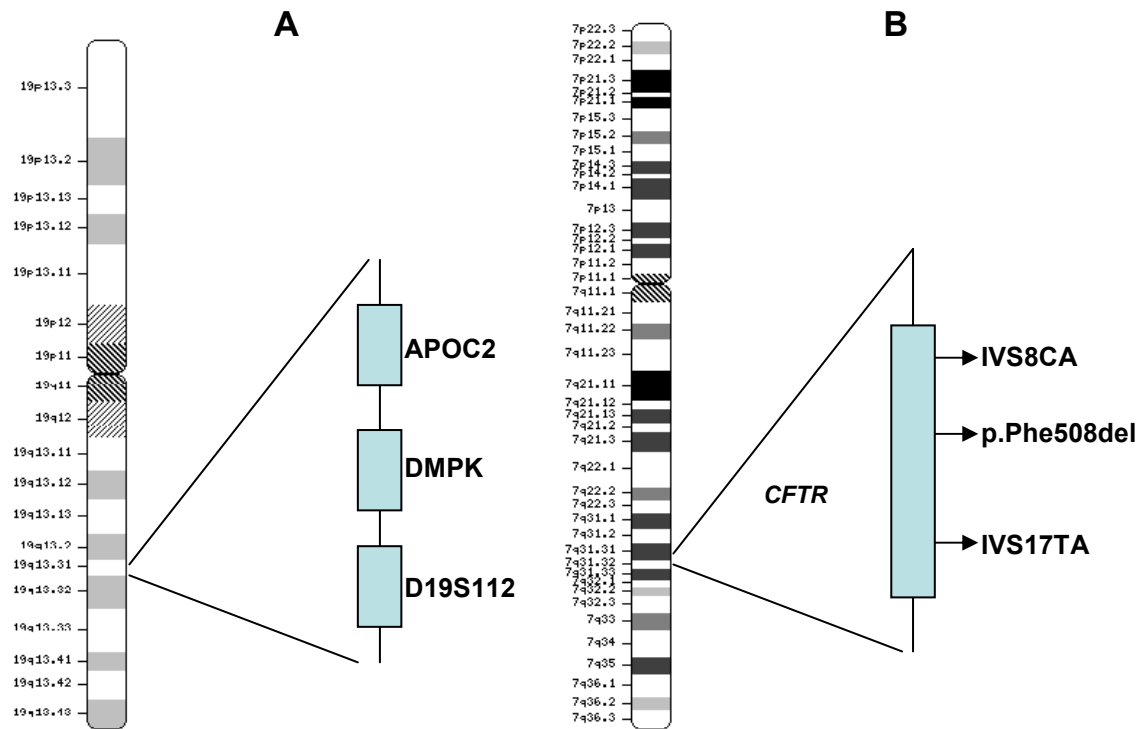
The main disadvantage encountered with Phi29 polymerase is the requirement of a high quality of DNA in order to obtain best results (Hughes et al., 2005; Lage et al., 2003). Different groups have shown the only underrepresented regions of the genome are those in the proximity of the duplex ends which are usually abundant in degraded and damaged DNA (Panelli et al., 2006). MDA did not perform as expected in samples where the starting material was from lower molecular weight, such as paraffin-embedded clinical samples and deteriorated forensic samples (Wang et al., 2004; Lage et al., 2003).

In single cell studies the quality of DNA is largely dependent on the cell type and cell lysis used in order for MDA to have access to the DNA. In PGD different cell types such as buccal cells and lymphocytes are routinely used to design and optimise new PGD protocols prior to application to blastomeres (Thornhill et al., 2005). Buccal cells are easily obtained from patients and are larger cells compared to lymphocytes but their DNA is considered of poor quality as they have entered the phase of apoptosis (Piyamongkol et al., 2003). The quality of the DNA is also dependent on the cell lysis used to make the DNA accessible to the polymerase. Two different lysis methods have been widely used in single cells; alkaline lysis (ALB) and enzymatic lysis (proteinase K lysis). In this chapter three lysis buffers were used; proteinase K, a standard ALB and a modified ALB in which the DTT was excluded as it has been suggested to be harmful for DNA (Pierce et al., 2002). The different cell types and cell lysis were used in order to determine if and how MDA is influenced by these factors at the single cell level.

The optimisation and use of MDA as a universal step in PGD for the amplification of the entire genome prior to any molecular analysis would probably be the most efficient and reliable approach for PGD evolution. This is because designing multiplex PCR and optimising the reaction at the single cell level can be very laborious and expensive (Obradors et al., 2008; Henegariu et al., 1997). Usually, a PGD test is specific to the family and months of work are needed for optimisation at the single cell level.

Two widely used PGD tests, which are also used as a model here, are for myotonic dystrophy type I (DM I) and cystic fibrosis (CF). For DM I, the PGD protocol usually consists of a PCR product targeted on the specific Dystrophin myotonia-protein kinase (*DMPK*) causative gene and two linked markers (less than 1CM apart) in the 19q13.2-19q13.4 area (Kakourou et al., 2007). The reason for using two linked markers is in case of allele-drop out (ADO) and total amplification failure (TAF) events. Diagnosis can also be based from indirect analysis of these markers rather than the direct detection of the mutation marker. In this project APOC2 and D19S112 dinucleotide short tandem repeat (STR) markers were chosen because they have already been used in our clinical PGD lab and they have high heterozygosity (85.2% and 86.3% respectively). In normal individuals the heterozygosity level for the DM (trinucleotide repeat) marker is 72.9% but the pathogenic alleles are refractory to PCR since the expansion may include thousands of repeats (Kakourou et al., 2007). Interestingly, a crossover between the APOC2 and the DM marker has been described even though they are considered as linked markers illustrating the need for using more than one marker as confirmation of diagnosis (Kakourou et al., 2007). For the CF PGD test, the PCR product targets the p.Phe508del deletion, which represents the most common cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutation (a three basepair deletion) and gives a 94bp product for the normal allele and a 91bp for the mutant allele with the primers used in this project. Other STR markers used for the multiplex PCR are located in the introns or exons of the *CFTR* gene. Two dinucleotide STR markers chosen for this project were located in intron 17 and 18 of the *CFTR* gene (IVS17TA and IVS18CA) and have been used in clinical PGD in our laboratory (figure 3.1).





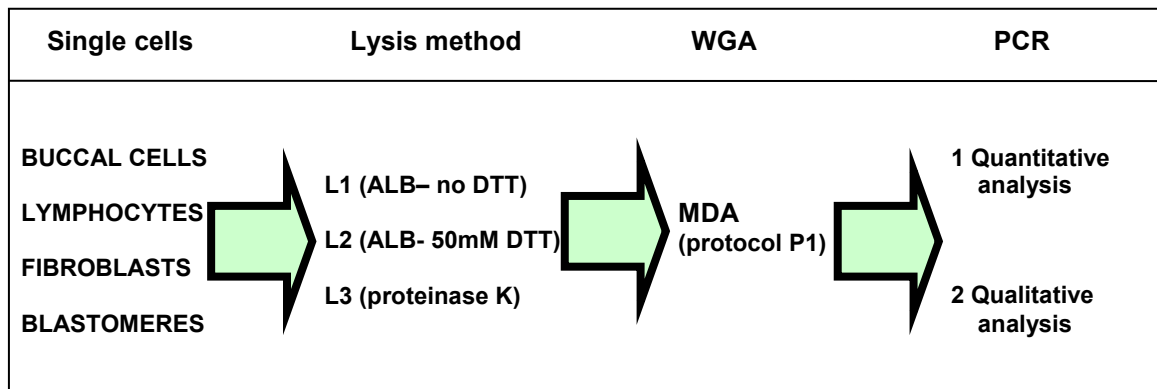
**Figure 3.1: Location of markers on chromosomes 19 and 7.** A: This diagram shows where and how the STR markers APOC2, DMPK and D19S112 are located and linked on chromosome 19. B: The dinucleotide repeats in intron 8 (IVS8CA) and 17 (IVS17TA) are shown and how they are located compared to the p.Phe508del mutation marker in the CFTR gene.

### 3.3 Aims

The aim of this study was to perform MDA on single cells and obtain reliable results from PCR. It was tested if different cells and cell types would have an affect on MDA at the single cell level.

### 3.4 Experimental Design

The molecular results were separated in to two sections, preliminary and main results. Preliminary results include basic experiments such as measuring the concentration of the DNA and the MDA products, determining the allele sizes obtained from the gDNA, testing the negative samples to avoid contamination (negative control) and obtaining the results from MDA products where the starting DNA template was gDNA (positive controls). In addition to these experiments, PCR was applied directly to single cells and to MDA products with different lysis methods. After the determination of the best lysis method, the main results include experiments with MDA products from different cell types. Qualitative analysis of the results was also performed that concentrate on the stutter patterns, the peak heights of the results, the ADO rate of the large/small alleles and the comparison of 25 and 40 PCR cycles. Figure 3.2 illustrates the experimental design of this project.



**Figure 3.2: Synopsis of the experimental design of this chapter.** The diagram shows how molecular analysis was performed on single cell MDA products from different cell types and lysis methods.

The experiments involved MDA products from single buccal cells, lymphocytes, fibroblasts and blastomeres that were lysed with the L1, L2 or L3 method (section 2.3), amplified with the P1 protocol (section 2.4.2, table 2.2) and subjected to triplex PCR. The analysis of the results was completed using specific measurements. These were the MDA amplification efficiency (MDA-AE), the allele drop out (ADO), the total amplification

failure (TAF) and the accuracy of the results. The MDA-AE was calculated as the number of MDA products that generated a PCR product for at least one of the markers tested. The ADO rate was calculated as the percentage of heterozygous loci that appeared to be homozygous following PCR of MDA products whereas the TAF rate was calculated as the percentage of MDA products that completely failed to amplify by PCR at a given locus. The accuracy rate was measured as the percentage of MDA products that gave the expected allele size (EAS). In the following colour tables, EAS appears as a green box, ADO as blue and TAF as black. In a few cases a red box is shown which indicates that an unexpected allele size appeared. Cells highlighted in black indicated that the MDA amplification had failed and so were excluded from post PCR analysis.

### **3.5 Materials and methods**

#### **3.5.1 DNA materials**

The DNA used for the molecular project was obtained from various sources. Two donors (A and B) provided blood samples from which DNA was extracted and single lymphocytes were collected and lysed as described in sections 2.2.1 and 2.2.3. The same individuals also provided buccal cells for single cell isolation and lysis. Skin fibroblast cultures that were grown in culture were obtained from two fetuses, one with trisomy 13 and the other with trisomy 21 (named C and D respectively). Single blastomeres were obtained after disaggregation of embryos and their DNA was obtained after single cell isolation and cell lysis. The embryos used for this project were from PGS patients that were non-transferable due to chromosomal abnormalities found in clinical work. As reported from the embryologists that did the biopsy, all the blastomeres used for the molecular project were obtained from day 5 embryos that were previously frozen and their quality was validated from 2CC to 4BC. Single blastomeres were collected after disaggregation of the embryos using standard PBS/spreading solution (Mantzouratou et al., 2009). Embryo handling and disaggregation was performed from Dr Anna Mantzouratou and all the work was performed in a DNA-free area which is specialised for single cell work. Table 3.1 summarises the number and the different types of cells

isolated for this chapter. The DNA from the single cells was subjected directly to PCR or it was amplified with MDA prior to PCR analysis.

From a total of 270 single cells isolated the majority were first amplified with MDA prior to molecular analysis. A total of 75 MDA products derived from single buccal cells (donors A and B), 75 MDA products derived from single lymphocytes (donors A and B), 20 MDA products derived from trisomic fibroblasts (C and D), 40 MDA products derived from single blastomeres, 50 single buccal cells and 50 single lymphocytes were used as the DNA source for the PCR reactions for the molecular project.

DNA SOURCE	gDNA	Single cells			
		BUCCAL CELLS	LYMPHOCYTES	FIBROBLASTS	BLASTOMERES
DONOR A	✓	80	80	X	X
DONOR B	✓	25	25	X	X
TRISOMY C	✓	X	X	10	X
TRISOMY D	✓	X	X	10	X
EMBRYOS (20)	From parents: Not always	X	X	X	34

**Table 3.1: Summary of DNA source used.** This table summarizes the DNA samples from various cell types for this chapter. The DNA from the parents of the embryos was not always available. Symbol “✓” was used to show that gDNA was obtained and symbol “X” was used to show that the specific types of cells were not obtained.

### 3.5.2 DNA concentrations

The DNA concentrations from the gDNA and MDA products were measured with the use of a spectrophotometer (ND-1000 UV-Vis, NanoDrop, USA) and its computer program (ND-1000 software, NanoDrop, USA).

### 3.5.3 Ethical approval

For the blastomeres ethical approval was obtained from the Human Fertilisation and Embryology Authority (HFEA). Written consent was obtained from the couples who donated their embryos.

### 3.5.4 Polymerase chain reaction

Two triplex fluorescent PCR (F-PCR) reactions were applied to assess the MDA efficiency and accuracy to amplify single cells (Appendix A6). Both multiplex reactions had been previously optimised for direct application on single cells and have been used in clinical PGD in our laboratory (Kakourou et al., 2007; Moutou et al., 2004). Each of the forward primers was fluorescently labeled with either Fam, Ned or VIC dye. The details for each primer including the fluorescent label and the heterozygosity level are shown in table 3.4. The primer sequences are listed in appendix A, table A1. HIFI polymerase (Roche Diagnostics Ltd, UK), 10x HIFI fidelity buffer II (Roche Diagnostics Ltd, UK), dNTPs (Promega, USA), glycerol (Sigma, UK) and nuclease free H<sub>2</sub>O (Promega, USA) were used for the PCR reaction. All PCR Master mixes were prepared in a DNA free room with positive pressure inside a hood. All PCR reactions were carried out in an ABI9700 thermocycler (PE Applied Biosystems, UK).

Loci	Type of sequence	Forward primer label	Region	Product size (bp)	Heterozygosity
DM1	Trinucleotide	VIC ®	19q13.3	120-203	72.9%
D19S112	Dinucleotide	NED <sup>TM</sup>	19q13.3-19q13.4	117-142	86.3%
APOC2	Dinucleotide	FAM ®	19q13.2	125-170	85.2%
p.Phe508del	Specific	FAM ®	7q31.2	94	-
IVS8CA	dinucleotide	NED <sup>TM</sup>	7q31.2	180-200	71%
IVS17TA	dinucleotide	FAM ®	7q31.2	180-280	89%

**Table 3.2: Details of the primers used for chapter 3.** This table summarizes the primers used for the two triplex PCR reaction, including the fluorescent label of the forward primer, the regions that amplify, the expected product size and the heterozygosity level.

#### **3.5.4.1 DM triplex**

The first multiplex PCR (DM triplex) was a triplex including three STR markers located in or around the area of the dystrophin (DMPK) gene (DM1, APOC2 and D19S112). The PCR reactions had a final volume of 25 $\mu$ l and consisted of 0.2mM dNTPs, 10% glycerol, 1x HiFi fidelity buffer II containing 20mM MgCl<sub>2</sub>, 1.5 units HiFi Polymerase, primer sets of 0.2 $\mu$ M DM1, 0.3 $\mu$ M APOC2 and 0.3 $\mu$ M D19S112, and nuclease free H<sub>2</sub>O. For MDA products and gDNA 1 $\mu$ l was added to the mixture whereas in single cells the volume containing the DNA was estimated to be 3 $\mu$ l.

The following conditions were used in the ABI9700 thermocycler for the DM triplex: Initial denaturation at 96°C for two minutes followed by 94°C for 15 seconds (96°C for the first 10 cycles), annealing at 58°C for 45 seconds and extension at 72°C for one minute for 25-40 cycles. After a final elongation step at 72°C for 7 minutes the samples were stored at 4°C until used. For single cells and MDA products 40 cycles were used whereas 25 cycles were used for some MDA products from single cells in order to compare the difference in the cycle number.

#### **3.5.4.2 CF triplex**

The second multiplex (CF triplex) was a triplex containing three markers on the cystic fibrosis transmembrane conductance regulator (CFTR) gene (p.Phe508del, IVS8CA, IVS17TA). For the CF triplex, the reaction for single cells was performed as a split reaction because the last two markers interacted with each other. The reagents in the first round were similar to the DM triplex with the difference being the primer sets (0.2 $\mu$ M p.Phe508del, 0.6 $\mu$ M IVS8CA and 0.6 $\mu$ M IVS17TA). For the second round, 5 $\mu$ l of the PCR reaction from the first round was as the DNA template. The PCR reagents were only different in the primer sets where in one tube 0.2 $\mu$ M p.Phe508del and 0.6 $\mu$ M IVS8CA were added and in the other tube only 0.6 $\mu$ M IVS17TA was added. The PCR conditions in the first round included an initial denaturation at 96°C for two minutes, 10 cycles with denaturation at 96°C for 15 seconds, annealing at 51°C for 45 seconds and extension at

72°C for one minute and a final elongation step at 72°C for 7 minutes. For the second round the conditions were the same but the cycles were 25 instead of 10 and the denaturation step was set at 94°C instead of 96°C.

For gDNA and MDA products, the triplex reaction was separated from the beginning resulting in a duplex reaction (CF1) with the p.Phe508del and the IVS8CA markers and a singleplex reaction (CF2) with the IVS17TA marker. In the case of the duplex and the singleplex reaction (CF1 and CF2) the PCR reagents and conditions were identical to the ones used for the second round in the CF triplex.

### **3.5.5 PCR analysis**

The PCR products were run on the ABI PRISM 310 genetic analyser (single capillary). Separations for each product were performed at 15,000 volt for 30 minutes, at 60°C using the POP-6 sieving polymer (PE Applied Biosystems, UK) and 1 x genetic analyser buffer (PE Applied Biosystems, UK). Before the run, 1.5µl of the PCR product was mixed with 12µl of formamide (Sigma, UK) and 0.5µl of Genescan-500 ROX size standard (PE Applied Biosystems, UK) in 0.5 tubes which were capped with rubber septa. This specific capping was necessary for the PRISM in order to have access to the sample through the capillary. The samples were then denatured at 95°C for 4 minutes in a Thermal Reactor (Hybaid, UK) and transferred to the ABI PRISM for sequence detection. As one of the primers was fluorescently labelled at the 5' end with FAM (blue), VIC (green) or NED (black) the PCR products would be detected by the PRISM and the data were analysed using Genemapper analysis software version 3.5 (PE Applied Biosystems, UK).

### **3.5.6 Statistical analysis**

Statistical analysis of the experiments was performed in order to compare the results. Chi square test was used to test the statistical difference between different cell types and cell lysis methods. For statistical analysis of the results the EAS was taken into account.

## **3.6 Results**

### **3.6.1 Preliminary experiments**

#### **3.6.1.1 DNA concentration**

The concentration of gDNA ranged between 0.9 - 1.3 $\mu$ g/ $\mu$ l. The concentration of the MDA products from single cells and gDNA was calculated to vary from 0.5 to 2 $\mu$ g/ $\mu$ l (absorbance ratio: 1.38-1.6). This range of concentration included all MDA products, even those in which PCR analysis showed a failure of MDA amplification in all the loci and the negative sample. The MDA products were not purified but used directly for PCR experiments.

#### **3.6.1.2 Allele sizes**

The allele sizes from each DNA sample used were obtained from PCR on gDNA extracted from each individual. DNA from individual A and trisomy 21 (D) for the DM locus were the only fully informative markers. The p.Phe508del marker was the only non polymorphic marker and the same allele size is expected in all individuals lacking the p.Phe508del mutation. The results are shown in table 3.3. For the blastomeres, in two cases (E1, E2 and F1, F2) parental DNA was available for comparison of alleles with the embryo results (EB and FB embryos respectively). For the remaining four families gDNA was not available from the parents in order to confirm the expected allele sizes and so suggested sizes depended on comparison of results from blastomeres from the same embryo and from comparison of blastomeres from embryos from the same parents.



gDNA	DM markers			CF markers		
	APOC2	DM1	D19S112	Phe508	IVS8CA	IVS17TA
A	152 154	140 155	123 132	94	180	200 250
B	127 150	122	117 134	94	180	229 250
C	127 150	122	139 141	94	180 182	245 285
D	135 150	145 172	132 135	94	182 198	229 250
E1	148 152	142 148	117 136	-	-	-
E2	150 154	148 181	133 136	-	-	-
F1	148 150	136 148	133	-	-	-
F2	151 157	132 147	133	-	-	-

**Table 3.3: True allele sizes after PCR of gDNA.** The table shows the allele sizes on six markers of the two individuals (A and B), the two trisomic fibroblast cells (C and D) and from DNA from the parents (E1, E2, F1 and F2) of the embryos. Symbol (-) is used to show that PCR analysis was not done for the CF markers.

### 3.6.1.3 Negative and positive controls for MDA

During single cell isolation, for each cell that was isolated a negative control was processed in order to check for contamination during the isolation process. All negative controls were subjected directly to PCR for the DM locus rather than being amplified first by MDA. This assessed the absence of contamination in a cost-effective, reliable and fast way. None of the negative controls showed any relevant or irrelevant peaks indicating the absence of contamination in all the single cells collected. A negative PCR control was also included in each experiment where deionised nano-pure H<sub>2</sub>O substituted DNA. All negative PCR controls showed no contamination. A negative MDA sample was subjected to PCR for the DM and the CF locus in order to check if any artefacts would appear from nonspecific DNA produced by the MDA reaction itself. Most of the artefacts produced were less than 120bp long and their peak heights were very low (100 – 300 units) compared to the real peaks (500 – 9000 units) from the MDA products from the single cells. For each PCR experiment, positive controls included both gDNA and MDA products from gDNA in order to confirm the accuracy and the efficiency of the reaction. Six MDA products from gDNA of individuals A and B were produced as positive controls with 100% MDA-AE, 100% EAS, 0% ADO and 0% TAF.

#### **3.6.1.4 Single cell PCR**

PCR for the DM triplex was applied to single lymphocytes and buccal cells as in clinical PGD workups. L1 (ALB lysis without DTT) and L3 (proteinase K lysis) methods were used. The results are shown in tables 3.4 and 3.5 and figure 3.3. The cells that are highlighted in black were the ones where PCR did not work and were excluded from post PCR analysis. PCR efficiency was calculated to be 90.0% (27/30) for all cell types and lysis methods. PCR failure may be due to failure of the reaction but also due to loss of the cell during transfer. The results show that the L1 lysis method gave more accurate results compared to the L3 lysis method in both cell types (72.8% (59/81) compared to 65.1% (43/66) in buccal cells and 92.6% (75/81) compared to 47.0% (31/66) in lymphocytes). Chi square analysis showed that the results from the different lysis methods were not statistically different for the EAS in buccal cells ( $p = 0.169$ ) but were on lymphocytes ( $p < 0.001$ ). Lymphocytes that were lysed with L1 lysis provided more reliable results compared to the other lysis/cell type combination. Interestingly, lymphocytes lysed with the L3 method gave the worst results (47.0% accuracy, 24.2% ADO and 28.8% TAF) which indicated that enzymatic lysis was not suitable for lymphocytes. For buccal cells the major problem that L3 lysis accounted for was the high TAF (19.7%, 13/66) compared to L1 lysis (9.9%, 8/81) rather than ADO where it is almost the same (15.1% (10/66) and 17.3% (14/81) respectively). In lymphocytes TAF and ADO were much higher when using L3 lysis (28.8% and 24.2% respectively) compared to L1 lysis (2.5% and 4.9% respectively).

#### **3.6.1.5 MDA using the L3 method**

Since L3 (proteinase K) lysis was shown to produce the most inaccurate results in single cell PCR only five MDA products from single lymphocytes and buccal cells were produced initially and applied on the DM and CF triplex. MDA products from buccal cells using the L3 lysis had 26.7% (8/30) accurate results, 20.0% (6/30) ADO and 53.3% (17/30) TAF. MDA products from lymphocytes using the L3 lysis had 23.3% (7/30) accurate results, 3.3% (1/30) ADO and 70.0% (21/30) TAF. There was also one product

A: Buccal L1 lysis				B: Buccal L3 lysis				C: Lymphocytes L1 lysis				D: Lymphocytes L3 lysis			
DM TRIPLEX				DM TRIPLEX				DM TRIPLEX				DM TRIPLEX			
CELL NO.	APOC2	DM	D19S112	CELL NO.	APOC2	DM	D19S112	CELL NO.	APOC2	DM	D19S112	CELL NO.	APOC2	DM	D19S112
A1				A1				A1				A1			
A2				A2				A2				A2			
A3				A3				A3				A3			
A4				A4				A4				A4			
A5				A5				A5				A5			
A6				A6				A6				A6			
A7				A7				A7				A7			
A8				A8				A8				A8			
A9				A9				A9				A9			
A10				A10				A10				A10			
A11				A11				A11				A11			
A12				A12				A12				A12			
A13				A13				A13				A13			
A14				A14				A14				A14			
A15				A15				A15				A15			
A16				A16				A16				A16			
A17				A17				A17				A17			
A18				A18				A18				A18			
A19				A19				A19				A19			
A20				A20				A20				A20			
A21				A21				A21				A21			
A22				A22				A22				A22			
A23				A23				A23				A23			
A24				A24				A24				A24			
A25				A25				A25				A25			
A26				A26				A26				A26			
A27				A27				A27				A27			
A28				A28				A28				A28			
A29				A29				A29				A29			
A30				A30				A30				A30			

**Table 3.4: Triplex PCR on single buccal cells and lymphocytes after L1 or L3 lysis.** The table shows the results from single cell PCR for the DM locus on buccal cells and lymphocytes comparing the different lysis methods and cell types. Green boxes represent the expected allele size (EAS) whereas blue boxes show allele drop out (ADO) and black boxes show total amplification failure (TAF). Lymphocytes lysed with L1 method (C) provided the best results.

<b>Locus</b>	<b><i>Buccal cells L1 (n = 30)</i></b> <b><i>PCR-AE = 27/30 (90%)</i></b>			<b><i>Buccal cells L3 (n = 30)</i></b> <b><i>PCR-AE = 22/30 (73.3%)</i></b>			<b><i>Lymphocytes L1 (n = 30)</i></b> <b><i>PCR-AE = 27/30 (90%)</i></b>			<b><i>Lymphocytes L3 (n = 30)</i></b> <b><i>PCR-AE = 22/30 (73.3%)</i></b>		
	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>
<i>APOC2</i>	22/27(81.4)	1/27(3.7)	4/27(14.8)	16/22(72.7)	2/22(9.1)	4/22(18.2)	25/27(92.5)	1/27(3.7)	1/27(3.7)	17/22(77.3)	4/22(18.2)	1/22(4.5)
<i>DM</i>	17/27(62.9)	6/27(22.2)	4/27(14.8)	13/22(59)	2/22(9.1)	7/22(31.8)	25/27(92.5)	1/27(3.7)	1/27(3.7)	6/22(27.3)	10/22(45.5)	6/22(27.3)
<i>D19S112</i>	20/27(74.1)	7/27(25.9)	0/27(0)	14/22(63.6)	6/22(27.3)	2/22(9.1)	25/27(92.5)	2/27(7.4)	0/27(0)	8/22(36.4)	2/22(9.1)	12/22(54.5)
<b><i>TOTAL</i></b>	59/81(72.8)	14/81(17.3)	8/81(9.9)	43/66(65.1)	10/66(15.1)	13/66(19.7)	75/81(92.6)	4/81(4.9)	2/81(2.5)	31/66(47)	16/66(24.2)	19/66(28.8)

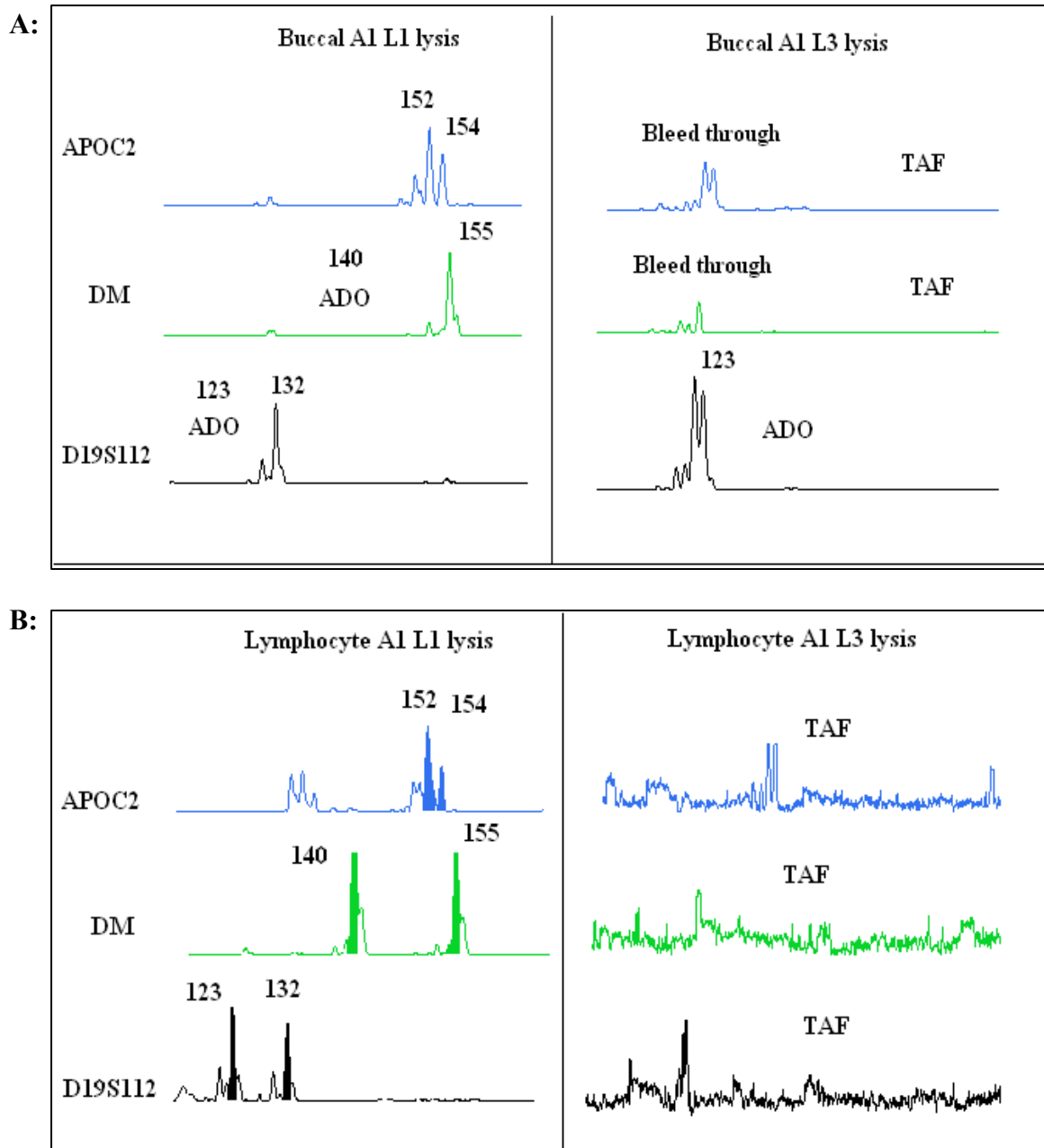
**Table 3.5: Summary of PCR analysis of single cells lysed with the L1 and L3 methods.** Single buccal cells and lymphocytes from individual A with two lysis methods (L1 and L3) are compared using an optimised triplex PCR for single cells. Better results are obtained from lymphocytes L1 lysis (92.6%) EAS) and worse results are obtained from lymphocytes L3 lysis (47%). This shows that cell lysis can play a key role according to the cell type that is used.

<sup>a</sup> EAS; expected allele size

<sup>b</sup> ADO; allele drop out

<sup>c</sup> TAF; total amplification failure

<sup>d</sup> All loci were homozygous.



**Figure 3.3: Electropherograms of single cell PCR for the DM triplex.** The figures show how the electropherograms look like after single cell PCR of buccal cells (A) and lymphocytes (B) for the DM triplex loci. The results on the left side were single cells lysed with L1 lysis whereas the results on the right side were single cells lysed with L3 lysis.

size that did not match the expected allele sizes. The high TAF in both cell types was the inhibiting parameter that showed that L3 lysis may not be useful for MDA and no further experiments with L3 lysis were made for MDA reactions. The results are shown in table 3.6 and figure 3.4. No statistical analysis was performed for MDA products and L3 lysis.

A:

MDA on single Buccal cells L3 lysis

DM TRIPLEX

CF TRIPLEX

CELL NO.

APOC2

DM

D19S112

phe508

IVS8CA

IVS17TA

A1

A2

A3

A4

A5

B:

MDA on single Lymphocytes L3 lysis

DM TRIPLEX

CF TRIPLEX

CELL NO.

APOC2

DM

D19S112

phe508

IVS8CA

IVS17TA

A1

A2

A3

A4

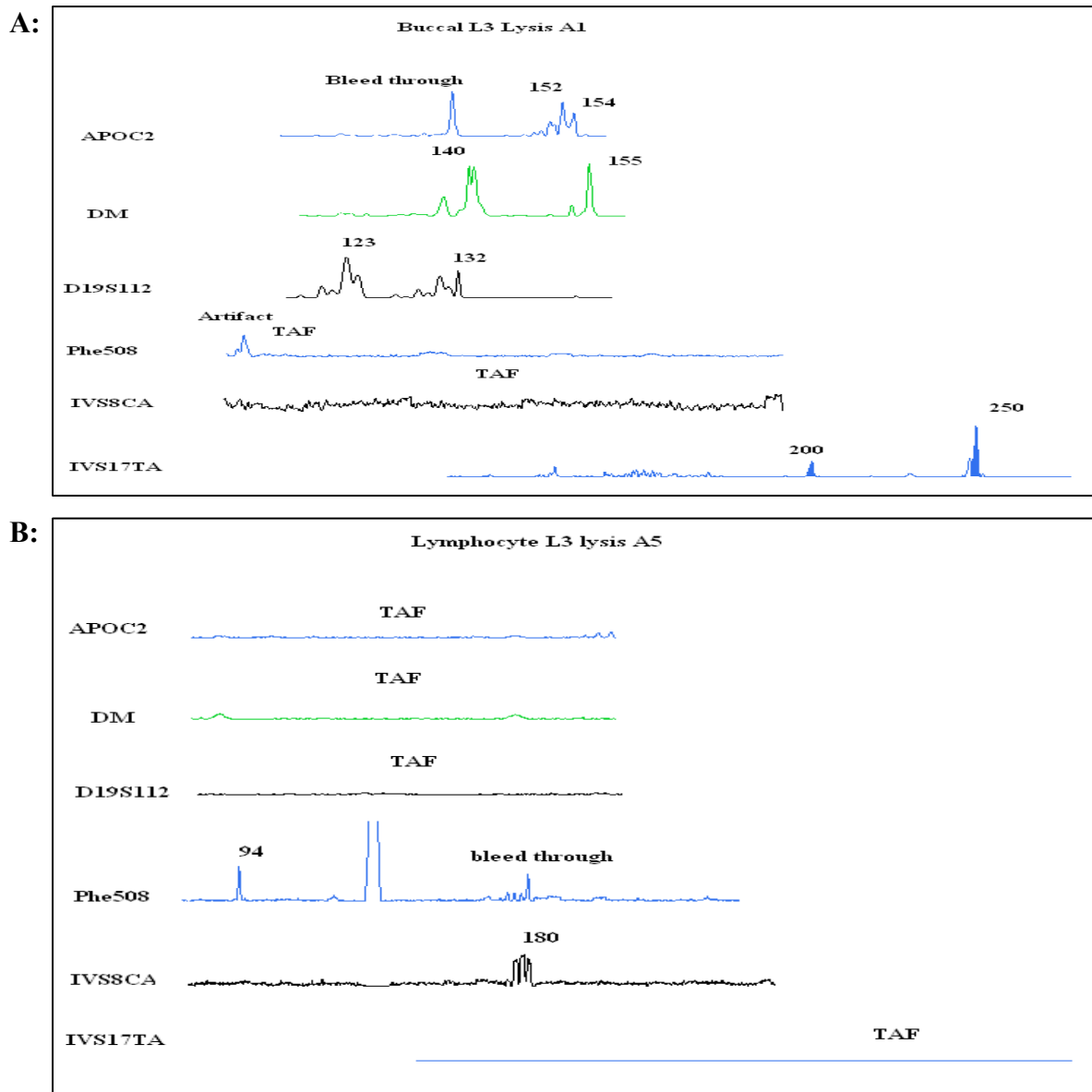
A5

**Table 3.6: Triplex PCR on MDA products from single buccal cells and lymphocytes after L3 lysis.** This table shows the results from DM and CF triplex PCR on MDA products from five single buccal cells (A) and lymphocytes (B). The red box shows that an incorrect allele size was detected on the DM locus. Green boxes represent the expected allele size (EAS) whereas blue boxes show allele drop out (ADO) and black boxes show total amplification failure (TAF).

### 3.6.1.6 MDA using the L1 and L2 methods

It has been suggested that DTT may not be beneficial for single cell lysis prior to PCR (Pierce et al., 2002). The effect of DTT in cell lysis prior to MDA was measured by comparing L1 and L2 lysis in lymphocytes and buccal cells. Tables 3.7 and 3.8 show the results of 80 MDA products. Figures 3.5 and show the electropherograms of two cells after L1 and L2 lysis respectively. In buccal cells, MDA-AE seems to be hampered by the addition of DTT (L2 lysis) in the lysis buffer but when excluding these cells the results showed a higher accuracy rate (53.6% (45/84) compared to 41.7% (45/108)) and lower TAF (23.8% (20/84) compared to 35.2% (38/108) whereas ADO was very similar (32.1% (18/56) and 30.6% (22/72)). A chi squared test showed that the lysis methods did not produce different results ( $p=0.101$ ) but the MDA-AE was much lower in the L3 lysis. For lymphocytes the L1 lysis method seemed to be beneficial for the MDA reaction since the EAS was higher and the TAF was lower even though there was no statistical difference ( $p=0.586$ ). The ADO rate was similar for both lysis methods. This means that ADO was not influenced by the lysis method but the biggest difference was seen on the

TAF and the MDA-AE. Results from L1 lysis were at least equal, if not beneficial, over L2 lysis for both cell types and so it was used in the following experiments.



**Figure 3.4: Electropherograms of single cell MDA products after L3 lysis.** The figures show the electropherograms of single buccal cells MDA (A) and single lymphocytes MDA (B) for the DM and CF triplex loci.

A: Buccal Cells L1 lysis

CELL NO.	DM TRIPLEX			CF TRIPLEX		
	APOC2	DM	D19S112	phe508	IVS8CA	IVS17TA
A1	Green	Black	Green	Green	Green	Black
A2	Blue	Black	Blue	Green	Black	Black
A3	Blue	Black	Black	Green	Black	Blue
A4	Black	Blue	Black	Black	Black	Black
A5	Blue	Blue	Red	Black	Black	Black
A6	Black	Black	Black	Black	Black	Black
A7	Green	Blue	Green	Green	Green	Blue
A8	Green	Black	Black	Green	Black	Black
A9	Green	Black	Black	Green	Green	Blue
A10	Green	Black	Blue	Green	Black	Blue
A11	Black	Red	Blue	Black	Black	Black
A12	Red	Black	Black	Green	Black	Black
A13	Green	Blue	Blue	Green	Black	Black
A14	Green	Black	Black	Green	Black	Black
A15	Green	Blue	Green	Black	Black	Black
A16	Green	Blue	Green	Green	Green	Green
A17	Black	Black	Blue	Black	Black	Blue
A18	Green	Black	Green	Black	Black	Black
A19	Green	Black	Green	Green	Black	Black
A20	Green	Black	Green	Green	Black	Black

B: Buccal Cells L2 lysis

CELL NO.	DM TRIPLEX			CF TRIPLEX		
	APOC2	DM	D19S112	phe508	IVS8CA	IVS17TA
A1	Black	Black	Black	Black	Black	Black
A2	Green	Blue	Green	Black	Green	Blue
A3	Black	Black	Black	Black	Black	Black
A4	Green	Black	Blue	Black	Red	Green
A5	Black	Black	Black	Black	Black	Black
A6	Black	Black	Black	Black	Black	Black
A7	Blue	Green	Blue	Black	Black	Black
A8	Green	Black	Blue	Green	Green	Black
A9	Green	Black	Black	Green	Black	Black
A10	Black	Black	Black	Black	Black	Black
A11	Black	Black	Black	Black	Black	Black
A12	Black	Black	Black	Green	Green	Black
A13	Black	Black	Black	Black	Black	Black
A14	Blue	Green	Black	Green	Black	Blue
A15	Green	Blue	Blue	Green	Green	Black
A16	Green	Blue	Blue	Green	Black	Black
A17	Green	Blue	Blue	Black	Black	Black
A18	Green	Blue	Blue	Green	Black	Green
A19	Green	Blue	Blue	Green	Black	Black
A20	Green	Blue	Black	Black	Black	Blue

C: Lymphocytes L1 lysis

CELL NO.	DM TRIPLEX			CF TRIPLEX		
	APOC2	DM	D19S112	phe508	IVS8CA	IVS17TA
A1	Green	Black	Green	Green	Green	Blue
A2	Green	Black	Green	Green	Black	Black
A3	Green	Black	Green	Green	Green	Green
A4	Black	Black	Black	Black	Black	Black
A5	Black	Black	Black	Black	Black	Black
A6	Green	Blue	Green	Green	Green	Blue
A7	Blue	Black	Green	Green	Green	Blue
A8	Green	Black	Black	Green	Black	Black
A9	Green	Black	Black	Green	Green	Blue
A10	Black	Red	Black	Black	Black	Black
A11	Green	Blue	Black	Green	Black	Blue
A12	Green	Black	Black	Green	Black	Blue
A13	Green	Blue	Blue	Green	Black	Black
A14	Green	Black	Green	Green	Black	Black
A15	Green	Blue	Green	Black	Black	Blue
A16	Green	Blue	Green	Green	Green	Blue
A17	Green	Black	Blue	Green	Black	Blue
A18	Green	Black	Green	Black	Black	Black
A19	Green	Black	Green	Green	Black	Black
A20	Blue	Black	Green	Green	Black	Black

D: Lymphocytes L2 lysis

CELL NO.	DM TRIPLEX			CF TRIPLEX		
	APOC2	DM	D19S112	phe508	IVS8CA	IVS17TA
A1	Green	Black	Blue	Green	Green	Black
A2	Green	Black	Green	Green	Green	Blue
A3	Green	Black	Green	Green	Green	Green
A4	Green	Black	Green	Green	Green	Green
A5	Green	Black	Green	Green	Green	Green
A6	Black	Black	Black	Green	Black	Black
A7	Black	Blue	Black	Black	Black	Blue
A8	Black	Black	Blue	Green	Black	Black
A9	Green	Black	Green	Green	Green	Blue
A10	Green	Blue	Black	Green	Green	Black
A11	Black	Blue	Black	Black	Black	Black
A12	Green	Black	Blue	Black	Black	Blue
A13	Green	Black	Green	Green	Black	Blue
A14	Black	Blue	Black	Green	Black	Black
A15	Green	Blue	Blue	Black	Black	Black
A16	Green	Black	Black	Black	Black	Black
A17	Green	Black	Black	Green	Black	Black
A18	Blue	Green	Blue	Green	Black	Black
A19	Blue	Blue	Black	Black	Black	Green
A20	Green	Blue	Black	Black	Black	Black

**Table 3.7: PCR on MDA products from single buccal cells and lymphocytes after L1 and L2 lysis.** This table compares two different cell types (buccal cells and lymphocytes) and two lysis methods (L1 and L2) with the use of the two triplex PCR reactions. Green boxes represent the expected allele size (EAS) whereas blue boxes show allele drop out (ADO), black boxes show total amplification failure (TAF) and red boxes show inaccurate allele size. The inaccurate allele size was reported when the alleles obtained from the electropherograms did not match the expected alleles from the gDNA, so they could not be classified as ADO, EAS or TAF.



Locus	<i>Buccal cells L1 (n = 20)</i> <i>MDA-AE = 18/20 (90%)</i>			<i>Buccal cells L2 (n = 20)</i> <i>MDA-AE = 14/20 (70%)</i>			<i>Lymphocytes L1 (n = 20)</i> <i>MDA-AE = 19/20 (95%)</i>			<i>Lymphocytes L2 (n = 20)</i> <i>MDA-AE = 20/20 (100%)</i>		
	EAS (%) <sup>a</sup>	ADO (%) <sup>b</sup>	TAF (%) <sup>c</sup>	EAS (%) <sup>a</sup>	ADO (%) <sup>b</sup>	TAF (%) <sup>c</sup>	EAS (%) <sup>a</sup>	ADO (%) <sup>b</sup>	TAF (%) <sup>c</sup>	EAS (%) <sup>a</sup>	ADO (%) <sup>b</sup>	TAF (%) <sup>c</sup>
<i>APOC2</i>	12/18(66.7)	3/18(16.7)	2/18(11.1)	11/14(78.6)	2/14(14.3)	1/14(7.1)	16/19(84.2)	2/19(10.5)	1/19(5.3)	17/20(85)	1/20(5)	2/20(10)
<i>DM</i>	2/18(11.1)	8/18(44.4)	7/18(38.9)	8/14(57.1)	5/14(35.7)	1/14(7.1)	10/19(52.6)	6/19(31.6)	2/19(10.5)	13/20(65)	6/20(30)	1/20(5)
<i>D19S112</i>	7/18(38.9)	6/18(33.3)	4/18(22.2)	4/14(28.6)	7/14(50)	3/14(21.4)	16/19(84.2)	2/19(10.5)	1/19(5.3)	14/20(70)	5/20(25)	1/20(5)
<i>phe508</i>	13/18(72.2)	-	5/18(27.8)	8/14(57.1)	-	6/14(42.6)	18/19(94.7)	-	1/19(5.3)	15/20(75)	-	5/20(25)
<i>IVS8CA</i>	9/18(50)	-	9/18(50)	7/14(50)	-	6/14(42.6)	19/19(100)	-	0/19(0)	14/20(70)	-	6/20(30)
<i>IVS17TA</i>	2/18(11.1)	5/18(27.8)	11/18(61.1)	7/14(50)	4/14(28.6)	3/14(21.4)	10/19(52.6)	9/19(47.4)	0/19(0)	11/20(55)	6/20(30)	3/20(15)
<b>TOTAL</b>	45/108(41.7)	22/72(30.6)	38/108(35.2)	45/84(53.6)	18/56(32.1)	20/84(23.8)	89/114(78.1)	19/76(25)	5/114(4.3)	84/120(70)	18/80(22.5)	18/120(15)

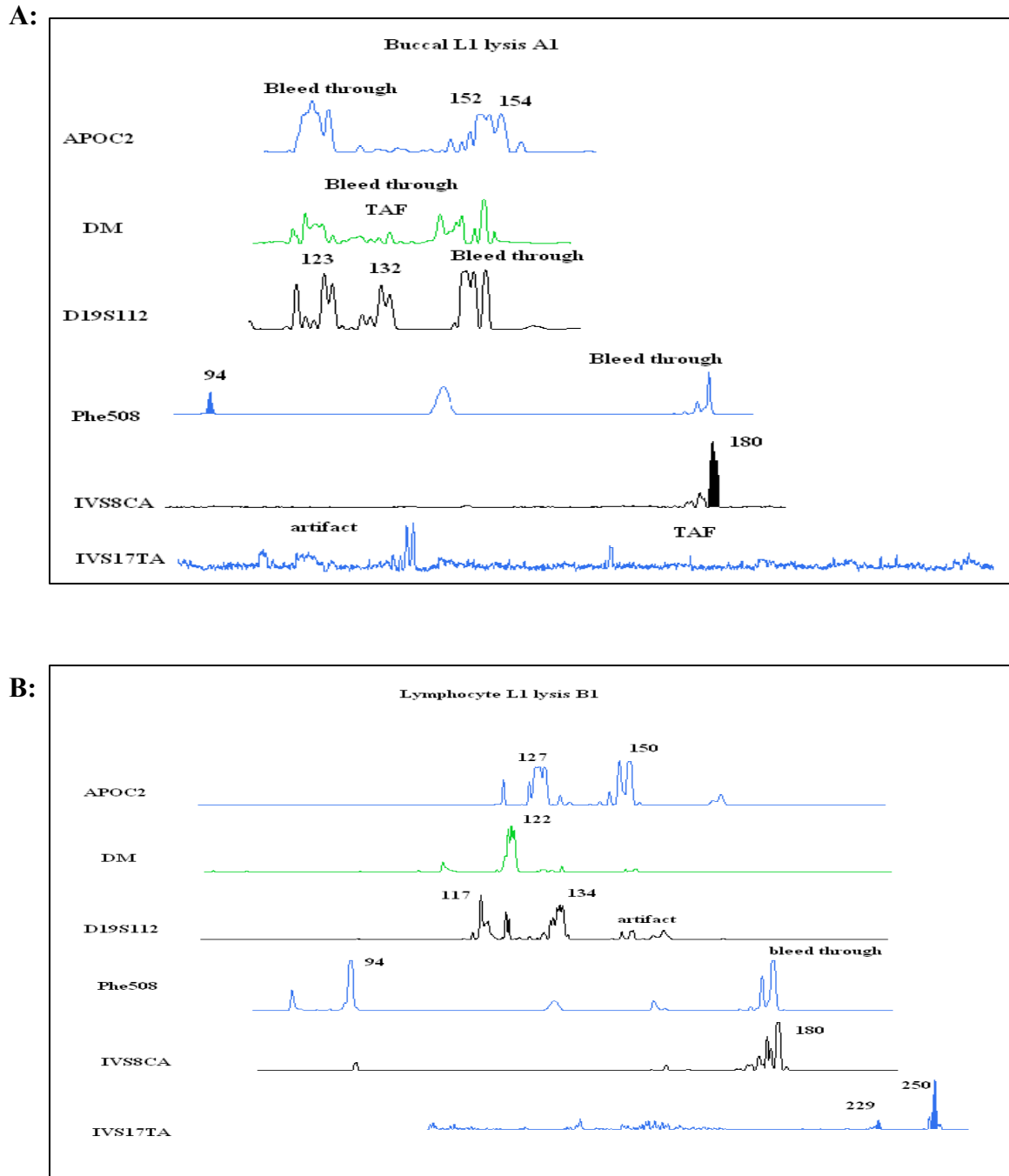
**Table 3.8: Summary of PCR analysis on MDA products lysed with L1 and L2 methods. L1 and L2 lysis methods are being compared for effectiveness on MDA reaction on two cell types and using two multiplex PCR reactions.**

<sup>a</sup> EAS; expected allele size

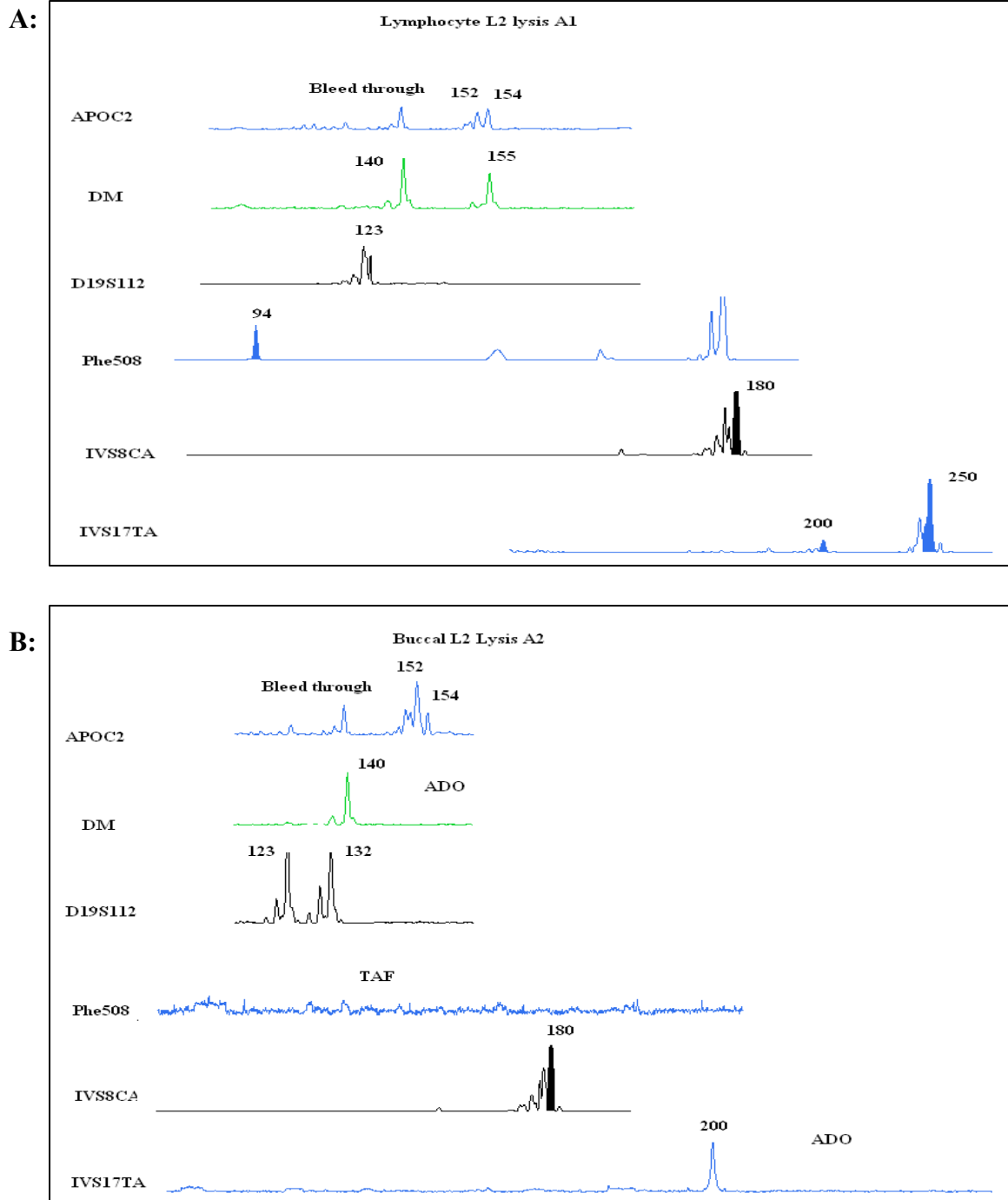
<sup>b</sup> ADO; allele drop out

<sup>c</sup> TAF; total amplification failure

<sup>d</sup> All loci were homozygote.



**Figure 3.5: Electropherograms of single cell MDA products after L1 lysis.** The figures show the electropherograms of single buccal cells MDA (A) and single lymphocytes MDA (B) after L1 lysis for the DM and CF triplex loci.



**Figure 3.6: Electropherograms of single cell MDA products after L2 lysis.** The figures show the electropherograms of a single buccal MDA (A) and a single lymphocyte MDA (B) after L2 lysis for the DM and CF triplex loci. For buccal cells the result A2 was used as no result was obtained from A1 buccal cell.

### **3.6.2 Main results**

#### **3.6.2.1 MDA using L1 lysis - Comparison of different cell types**

MDA using L1 lysis was applied to 50 buccal cells, 50 single lymphocytes and 20 fibroblasts prior to two multiplex PCRs for the DM and the CF loci. The results are shown in table 3.9. Electropherograms of the results of each cell type are shown on figure 3.7 The average ADO rate was 31.0% (45/145) for buccal cells, 20.8% (35/168) for lymphocytes and 20.0% (18/90) for fibroblasts but with high inter-locus variation (24.4 - 45.5% for buccal cells, 14.6 – 29.2% for lymphocytes and 5.0 – 30% for fibroblasts). MDA products from buccal cells showed very high TAF (22.0%, 54/245) compared with lymphocytes (6.6%, 19/288) and fibroblasts (7.5%, 9/120). Finally, following MDA 1.2% (3/245) and 0.3% (1/288) of the PCR products from buccal cells and lymphocytes respectively had an allele size that was did not match the expected allele sizes as determined from gDNA. A summary of the results is shown in Table 3.10.

It can be concluded that MDA products from single lymphocytes and fibroblasts produce similar results ( $p=0.396$ ) but significantly different from MDA products of single buccal cells ( $p<0.001$  with both cell types). For clinical PGD however, diagnostic work ups prior to blastomere diagnosis are being done on lymphocytes or buccal cells in order to optimise the PCR reaction. The best cell to do the work up would give comparable results to a blastomere. For this reason 34 blastomeres that were biopsied from 20 embryos and lysed with the same method (L1) were subjected to MDA prior to triplex PCR for the DM locus (Table 3.9D). CF triplex was not included in the blastomeres PCR as the heterozygosity level of IVS8CA and IVS17TA STR markers was not very high and so ADO events would have easily been misdiagnosed as EAS. The expected alleles of each embryo could only be based on the parental alleles and the results could not be 100% accurate unless the parents were fully informative for all the alleles. More markers could be used but then there could not be a direct comparison with buccal cells and lymphocytes. Unfortunately, gDNA from both parents was only available in the first 13 blastomeres (named as EB and FB) and they were not fully informative for all loci (Table

3.3, sample E1, E2, F1 and F2). For the rest of the blastomeres the results were based on comparing the allele sizes of blastomeres of the same embryos and blastomeres from siblings. The results are shown in table 3.9D. A comparison of the different cell types with blastomeres for the DM locus only is presented on table 3.11. Figure 3.8 shows the electropherograms of three blastomeres for the markers that it was tested. In two cases it cannot be concluded if a result was ADO or EAS and this was shown in the table as a green box with an X in the centre. These cases were excluded from post analysis. The embryos were expected to have chromosomal abnormalities as all the embryos were from PGS cases. MDA-AE was not calculated for blastomeres as three markers were not considered to be enough to make a secure outcome. The MDA products that did not show any result at all at three loci were excluded from analysis. The analysis shows a clear similarity of blastomeres with lymphocytes ( $p=0.904$ ) and fibroblasts ( $p=0.936$ ). However, the results of the electropherograms were more close to buccal cells as bleed through and artefacts were present in most of the results which made the diagnosis difficult.

A: Buccal Cells							
CELL NO.	DM TRIPLEX			CF TRIPLEX			
	APOC2	DM	D19S112	phe508	IVS8CA	IVS17TA	
A1							
A2							
A3							
A4							
A5							
A6							
A7							
A8							
A9							
A10							
A11							
A12							
A13							
A14							
A15							
A16							
A17							
A18							
A19							
A20							
A21							
A22							
A23							
A24							
A25							
B1							
B2							
B3							
B4							
B5							
B6							
B7							
B8							
B9							
B10							
B11							
B12							
B13							
B14							
B15							
B16							
B17							
B18							
B19							
B20							
B21							
B22							
B23							
B24							
B25							

B: Lymphocytes							
CELL NO.	DM TRIPLEX			CF TRIPLEX			
	APOC2	DM	D19S112	phe508	IVS8CA	IVS17TA	
A1							
A2							
A3							
A4							
A5							
A6							
A7							
A8							
A9							
A10							
A11							
A12							
A13							
A14							
A15							
A16							
A17							
A18							
A19							
A20							
A21							
A22							
A23							
A24							
A25							
B1							
B2							
B3							
B4							
B5							
B6							
B7							
B8							
B9							
B10							
B11							
B12							
B13							
B14							
B15							
B16							
B17							
B18							
B19							
B20							
B21							
B22							
B23							
B24							
B25							

*Table 3.9: Comparison of different cell types with L1 lysis method (continued on the following page).*

C: Fibroblasts						
CELL NO.	DM TRIPLEX			CF TRIPLEX		
	APOC2	DM	D19S112	Phe508	IVS8CA	IVS17TA
C1						
C2						
C3						
C4						
C5						
C6						
C7						
C8						
C9						
C10						
D1						
D2						
D3						
D4						
D5						
D6						
D7						
D8						
D9						
D10						

D: Blastomeres			
CELL NO.	DM TRIPLEX		
	APOC2	DM	D19S112
EB1.1			
EB1.2			
EB2.1			
EB3.1			
EB3.2			
EB4.1			
FB1.1			
FB1.2			
FB2.1			
FB3.1			
FB3.2			
FB4.1			
FB4.2			
GB1.1			
GB1.2			
GB2.1			
GB2.2			
GB3.1			
GB3.2			
GB4.1			
HB1.1			
HB1.2			
HB2.1			
HB2.2			
HB3.1			
IB1.1			
IB1.2			
IB2.1			
IB2.2			
JB1.1			
JB1.2			
JB2.1			
JB2.2			
JB3.1			

**Table 3.9: Comparison of different cell types with L1 lysis method.** This table shows the results from DM and CF triplex PCR on MDA products from single buccal cells (A), lymphocytes (B) and fibroblasts (C). and the results from DM locus from MDA products from single blastomeres (D). Four red boxes are present in the results that indicate the detection of incorrect allele size. Green boxes represent the expected allele size (EAS), blue boxes show allele drop out (ADO) and black boxes show total amplification failure (TAF). In table D two green boxes that contain a cross (X) were excluded from post analysis because it was impossible to conclude if ADO was present or not. In blastomeres 10 MDA products did not show any result which greatly decreases the MDA-AE comparing to MDA products from other cell types.

	<b><i>Buccal cells (n = 50)</i></b> <b><i>MDA-AE = 82% (41/50)</i></b>			<b><i>Lymphocytes (n = 50)</i></b> <b><i>MDA-AE = 96 % (48/50)</i></b>			<b><i>Fibroblasts (n = 20)</i></b> <b><i>MDA-AE = 100% (20/20)</i></b>		
<b>Locus</b>	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>
<i>APOC2</i>	26/41 (63.4)	10/41 (24.4)	4/41 (9.8)	39/48 (81.3)	7/48 (14.6)	2/48 (4.2)	18/20 (90)	1/20 (5)	1/20 (5)
<i>DM</i>	19/41 (46.3)	10/ 22 (45.5)	11/41 (24.3)	37/48 (77.1)	7/24 (29.2)	3/48 (6.3)	16/20 (80)	3/10 (30)	1/20 (5)
<i>D19S112</i>	20/41 (48.8)	15/41 (36.6)	5/41 (12.2)	39/48 (81.3)	7/48 (14.6)	2/48 (4.2)	14/20 (70)	5/20 (25)	1/20 (5)
<i>Phe508</i>	36/41 (87.8)	- <sup>d</sup>	5/41 (12.2)	46/48 (96)	- <sup>d</sup>	2/48 (4.2)	18/20 (90)	- <sup>d</sup>	2/20 (10)
<i>IVS8CA</i>	30/41 (73.1)	- <sup>d</sup>	11/41 (26.8)	46/48 (96)	- <sup>d</sup>	2/48 (4.2)	14/20 (70)	4/20 (20)	2/20 (10)
<i>IVS17TA</i>	14/41 (34.1)	10/41 (24.4)	17/41 (41.4)	26/48 (54.2)	14/48 (29.2)	8/48 (8.4)	13/20 (65)	5/20 (25)	2/20 (10)
<b>TOTAL</b>	145/246 (59)	45/145 (31)	53/246 (21.6)	233/288 (81)	35/168 (20.8)	19/288 (6.6)	93/120 (77.5)	18/90 (20)	9/120 (7.5)

**Table 3.10: Summary of the results of single cell MDA products from different cell types with L1 lysis.** The efficiency of single cell MDA on different cell types (buccal cells, lymphocytes and fibroblasts) was assessed. The four cases where an inaccurate allele was present were excluded from post analysis because of the minimum impact on the final results (1.2% for buccal cells and 0.3% for lymphocytes).

<sup>a</sup> EAS; expected allele size

<sup>b</sup> ADO; allele drop out

<sup>c</sup> TAF; total amplification failure

<sup>d</sup> All loci were homozygote.



	<b>Blastomeres (n = 34)</b>			<b>Buccal cells (n = 50)</b>			<b>Lymphocytes (n = 50)</b>			<b>Fibroblasts (n = 20)</b>		
<b>Locus</b>	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>	<b>EAS (%)<sup>a</sup></b>	<b>ADO (%)<sup>b</sup></b>	<b>TAF (%)<sup>c</sup></b>
<i>APOC2</i>	23/24(95.8)	1/16(6.25)	0/24	26/41 (63.4)	10/41 (24.4)	4/41 (9.8)	39/48 (81.3)	7/48 (14.6)	2/48 (4.2)	18/20 (90)	1/20 (5)	1/20 (5)
<i>DM</i>	15/24(62.5)	5/24(20.8)	2/24(8.3)	19/41 (46.3)	10/ 22 (45.5)	11/41 (24.3)	37/48 (77.1)	7/24 (29.2)	3/48 (6.3)	16/20 (80)	3/10 (30)	1/20 (5)
<i>DI9S112</i>	20/24(83.3)	1/18(5.5)	3/24(12.5)	20/41 (48.8)	15/41 (36.6)	5/41 (12.2)	39/48 (81.3)	7/48 (14.6)	2/48 (4.2)	14/20 (70)	5/20 (25)	1/20 (5)
<b>TOTAL</b>	58/72(80.5)	7/58(12.1)	5/72(6.9)	65/123(52.8)	35/104(33.7)	20/123(16.2)	115/144(80)	21/120(17.5)	7/144(4.9)	48/60(80)	9/50(18)	3/60(5)

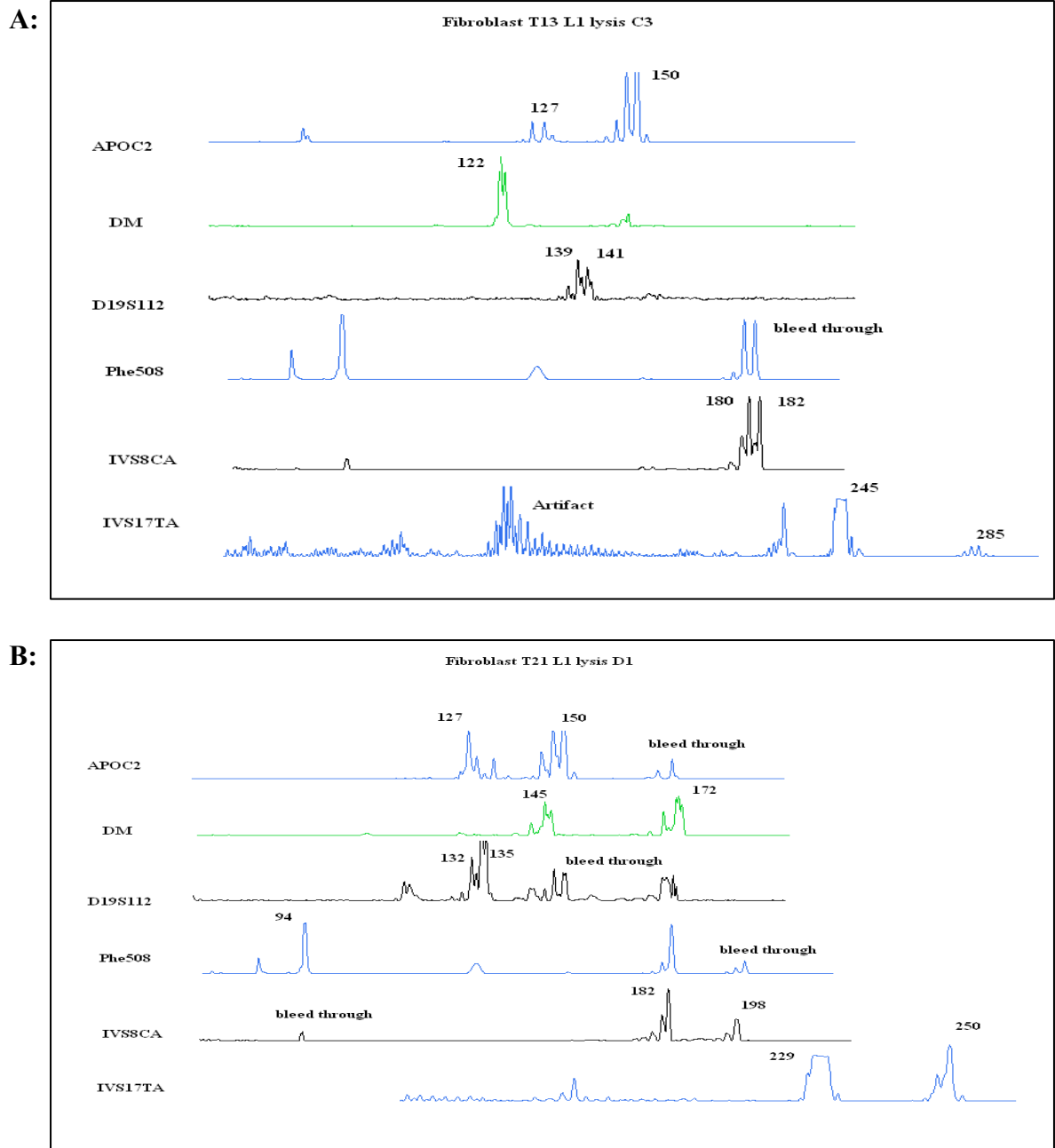
**Table 3.11: Summary of the results from MDA products from single blastomeres and comparison with other cell types.** This table compares the results obtained from blastomeres with the results of other cell types for the same loci. MDA-AE was not calculated as the results only from DM triplex were not considered enough for reliable analysis. For EAS, ADO and TAF calculations 10 MDA products were excluded because no results were obtained for any of the three markers. This could not be reliable concluded if it was from TAF or MDA amplification failure.

<sup>a</sup> EAS; expected allele size

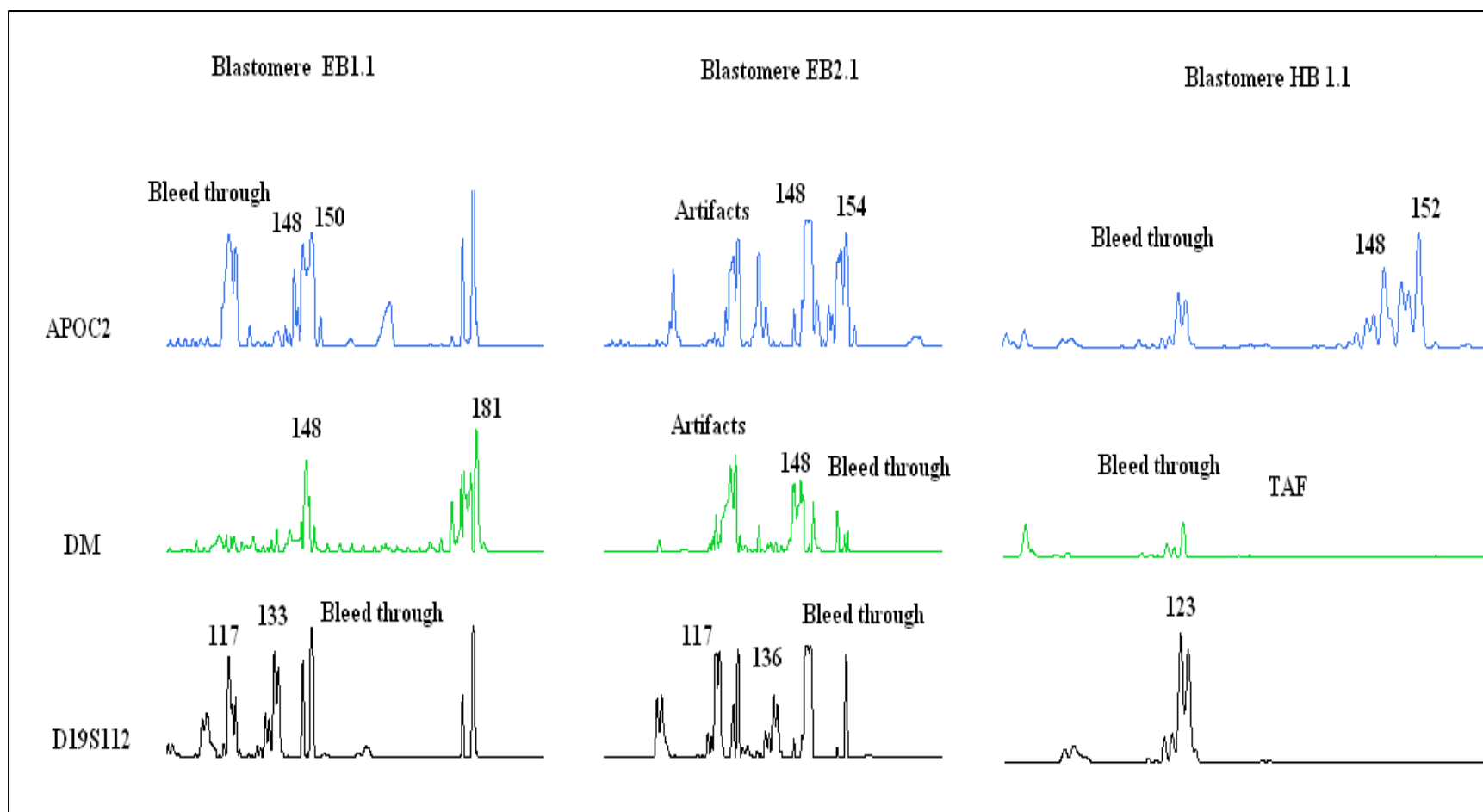
<sup>b</sup> ADO; allele drop out

<sup>c</sup> TAF; total amplification failure

<sup>d</sup> All loci were homozygote.



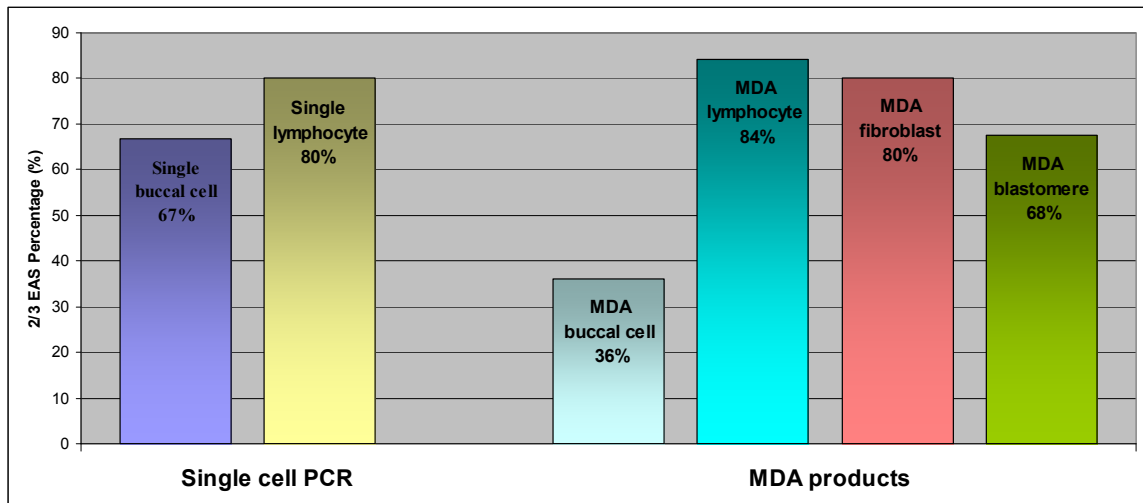
**Figure 3.7: Electropherograms of single cell MDA products after L1 lysis.** The figures show the electropherograms of one single fibroblast T13 MDA (A) and one single fibroblast T21 MDA (B) after L1 lysis for the DM and CF triplex loci.



**Figure 3.6: Electropherograms of single blastomeres MDA products after L1 lysis.** The figures show the electropherograms of three single blastomeres MDA after L1 lysis for the DM and CF triplex loci. The difficulty of the analysis was due to the many artefacts and bleed through present in the electropherograms.

### 3.6.2.2 Combined results of the triplex reaction.

Triplex PCR is used in clinical PGD in order to produce accurate diagnosis by overcoming problems such as ADO and TAF. For the DM and the CF triplex PCR, even if there was ADO or TAF in one marker, a secure diagnosis could be drawn for the embryo if the other two markers gave EAS. The diagnosis rate of PCR post MDA on different cell types was compared to single cell PCR without MDA. In clinical PGD diagnosis can be made when 2/3 markers show EAS. Figure 3.3 demonstrates the efficiency for diagnosis for each cell type lysed with L1 lysis for the DM triplex and compares it with direct triplex on single cells. In the case of MDA products from lymphocytes and buccal cells only the products from individual A were used. That was done in order to compare cells from the same individual and because individual B was not heterozygous for all loci (homozygous for the DM I locus). CF triplex was excluded from such comparisons as only one of the three markers was heterozygous. The figure shows that although single cell PCR gives higher EAS values overall there was a similar result when combining the results from the three markers.

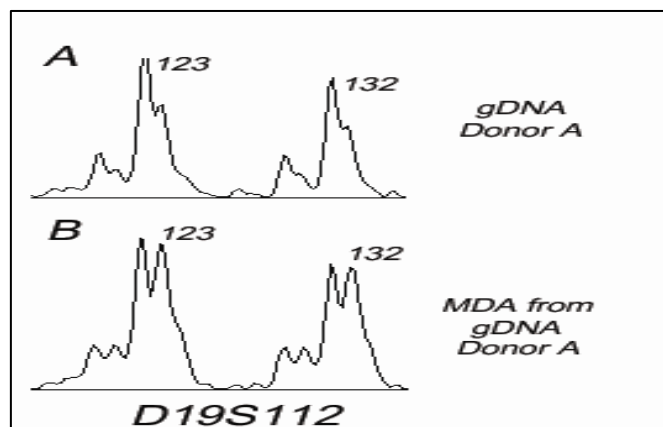


**Figure 3.8: Efficiency of diagnosis for DM triplex from different cell types with and without MDA.** This figure compares the combined results from the triplex PCR on the DM locus on single cells and MDA products. Buccal cells show a lower efficiency of diagnosis with or without the MDA application. Blastomeres post MDA show greater efficiency of diagnosis over MDA products from single buccal cells but lower compared to MDA products from single lymphocytes and fibroblasts. This result is largely due to the low MDA-AE.

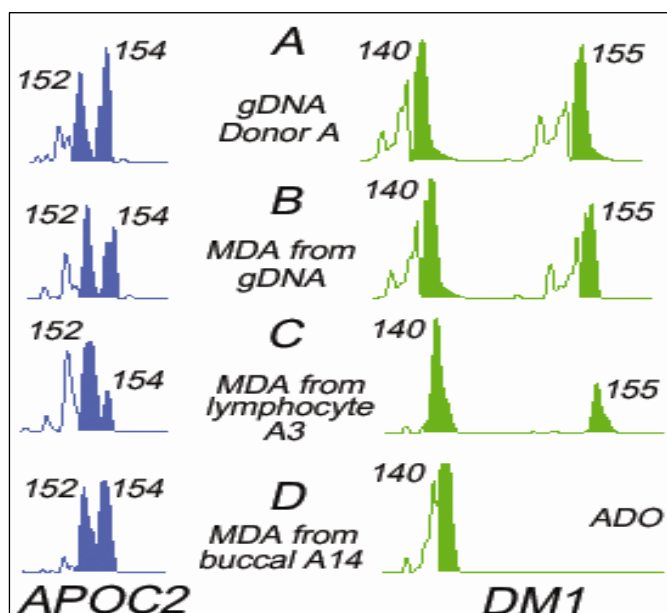
### 3.6.3 Qualitative analysis

#### 3.6.3.1 MDA effect on stutter bands, preferential amplification and ADO

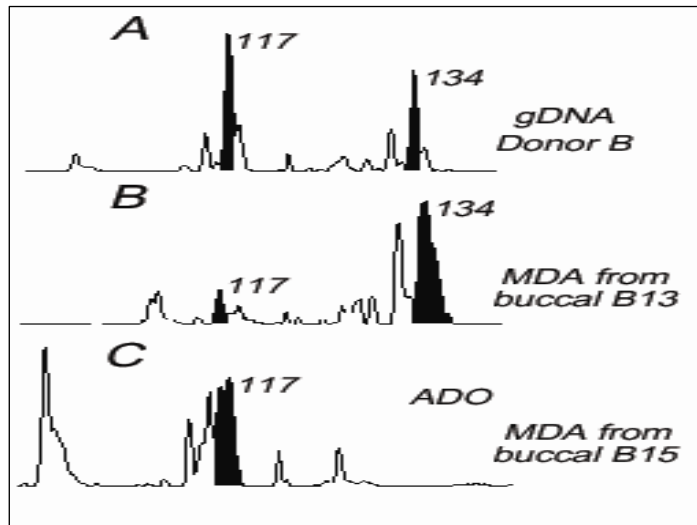
F-PCR analysis is mainly used for the detection of the allele length. Characteristic stutter patterns are associated with each STR locus. The examination of stutter bands and peak heights of the stutters is important in order to distinguish two alleles of similar size. This is because the additive effects of the stutter peaks alter the characteristic peak height ratios at that locus. The effect of MDA on the stutter bands and peak heights was examined in order to conclude if these parameters should be considered for diagnosis. PCR of MDA products from gDNA retained the same overall stutter pattern as that obtained from direct PCR of gDNA. Occasionally the stutter peaks were disproportionately high compared to the main peak (figure 3.4). Overall, complications of the stutter peak pattern were most pronounced in buccal cells. In some cases, involving MDA products from single cells, the pattern changed completely with the absence (figure 3.5D) or the presence of extra (figure 3.6B) stutter peaks and with the presence of stutter peaks with higher amplitude than the main peaks. Preferential amplification (the ratio between two peak heights in a heterozygote locus being  $> 3$  as described by Spits et al., 2006) was very common (61.8% for the DM I locus) in MDA products from single cells (figure 3.6B). These findings may support the conclusion that diagnosis or haplotyping on MDA products from single cells cannot depend on peak height or stutter patterns. PCR of four MDA products from single buccal cells and lymphocytes resulted in inaccurate allele sizes (figure 3.7 and 3.8). However for figure 3.7B the inaccurate allele size could also be considered as an extreme form of a change of a stutter band in height and size in combination with ADO of the larger allele. Overall, in 58.1% (57/98) of the ADO events in MDA products from single cells, the larger allele was absent. However, when heterozygous loci involving alleles differing by only two base pairs were excluded from the calculations the ADO rate of the larger alleles was calculated to be 50.6% (41/81).



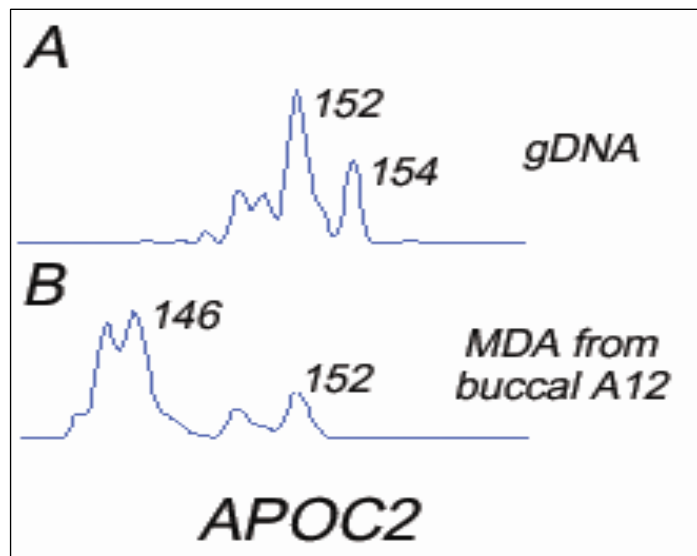
**Figure 3.9: Qualitative analysis of MDA from gDNA.** Comparison of the PCR result from D19S112 marker obtained from gDNA (A) and MDA product from gDNA (B). It is obvious that the stutter pattern remains the same but the stutters were greatly enlarged and had the same size as the original peaks.



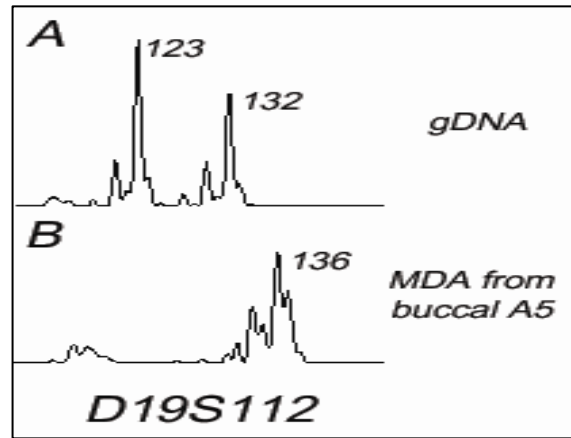
**Figure 3.10: Comparison of PCR results of MDA products from different cell types with gDNA.** This figure compares the PCR results from APOC2 and DM1 markers from gDNA (A), MDA product from gDNA (B), MDA product from a single lymphocyte (C) and MDA product from a single buccal cell (D). It is clear that the stutter pattern of the MDA products from the single lymphocyte and the buccal cell completely changes. ADO was also detected on this MDA product from this buccal cell.



**Figure 3.11: Electropherogram from MDA buccal cells and gDNA for the D19S112 locus.** Image A is the result from gDNA which is compared to image B (shows an extreme form of preferential amplification) and image C (shows ADO of the large allele). Images B and C also show how complicated and noisy the stutter patterns can be post MDA.



**Figure 3.12: Presence of inaccurate allele and ADO.** Electropherogram comparing the PCR result from the MDA product from buccal cell A12 (B) and from gDNA (A) of the APOC2 marker. The MDA product has ADO of the large allele (154) and also displays an inaccurate allele (146). The inaccurate allele could also be explained as an extreme form of stutter pattern and peak height change.



**Figure 3.13: Presence of inaccurate allele size.** This electropherogram comparing the PCR result from gDNA (A) and the MDA product from single buccal cell A5 on the D19S112 locus (B). The MDA product has ADO of the small allele and 3bp difference of the large allele compared to the gDNA result.

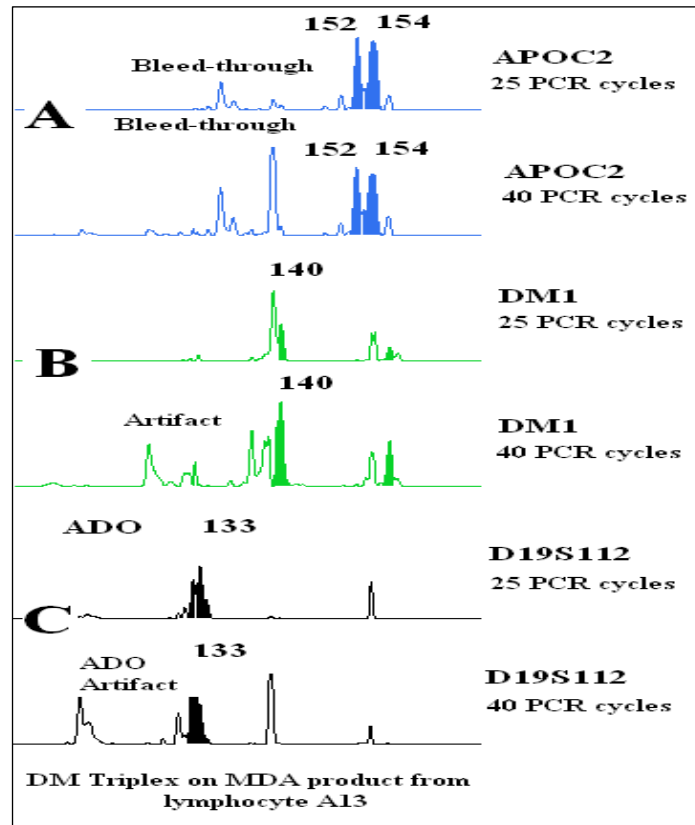
### 3.6.3.2 Comparison of 25 and 40 PCR cycles on MDA products

In single cell PCR, 35 - 40 cycles are usually needed for a sufficient amplification of the area of interest whereas for PCR on gDNA 25-30 cycles are usually enough for analysable results. The number of optimal PCR cycles on MDA products from single cells was investigated. This was achieved by repeating PCR for the DM locus on 20 MDA products from single lymphocytes and buccal cells with the difference of having 25 PCR cycles instead of 40. The results were compared with PCR results on the same MDA products when using 40 cycles. In theory, a decrease of the PCR cycles could reduce the production of unspecific peaks and decrease the noise of the results.

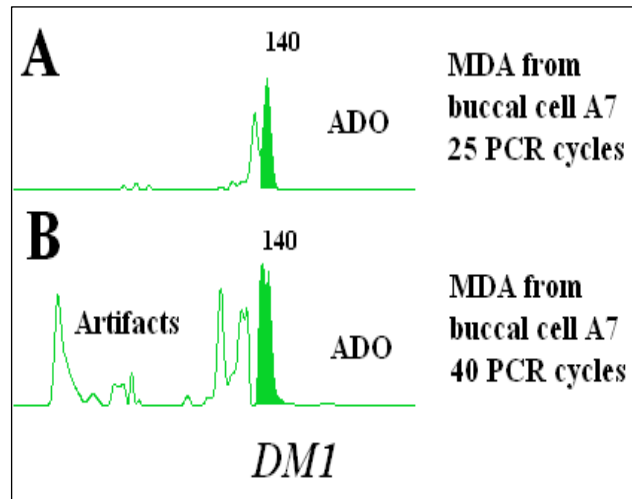
A comparison of the results obtained from 25 and 40 cycles on the same MDA products from single cells are shown on figures 3.9 and 3.10. These experiments were performed with cells with L1 lysis method. Overall, the reduction of the PCR cycles on the MDA products followed a very similar pattern. The main difference was that the peak height of the background noise, the stutter bands and the bleed-through effect were decreased when fewer cycles were used. Bleed-through effect is defined as the presence of excessive fluorescence of one labelled product being detected in the fluorescence of another dye



because of overlapping wavelengths of the two dyes. An example of bleed-through effect is when in multiplex PCR with three labelling dyes a PCR product that is labelled with VIC (green colour) is also detected in the analysis of the PCR product that is labelled with FAM (blue colour). An example of bleed-through is shown on figure 3.8A. Reducing the PCR cycles would also decrease the actual peak heights and this would increase the risk of misdiagnosis in case the peak height does not exceed 100 units (100 units is the limit for identifying a real peak, Figure 3.9B).



**Figure 3.9: Comparison of results of the same MDA product after different PCR cycles.** The electropherograms compare the DM triplex after 25 and 40 PCR cycles on MDA product from lymphocyte A13 (A: APOC2 locus, B: DM1 locus and C: D19S112 locus). It was obvious that bleed-through and artefacts were more visible on the 40 PCR cycles but overall the stutter pattern remained the same. On diagram B, the large allele of the 25 PCR cycle was underrepresented compared with the 40 PCR cycles and could be misinterpreted as ADO. Bleed-through of the DM I labelled product to the APOC2 electropherogram is detected. Blue colour: FAM label, Green colour: VIC label, Black colour: HEX label.



**Figure 3.10: Extreme presence of artefacts in more PCR cycles.** Comparison of (A) 25 and (B) 40 PCR cycles of DM locus on MDA products from buccal cell A7. ADO was present in both reaction but the 40 PCR cycles reactions exhibited too many artefacts that make the interpretation of the result unsecure and less clear.

### 3.7 Discussion

The limited amount of DNA is one of the greatest problems in a number of genetic tests, especially in PGD. WGA may play a crucial role in overcoming this problem but accurate and detailed research has to be done in order to prevent inaccurate diagnosis. In the limited number of publications regarding single cell MDA and PCR analysis a large variation in the results has been reported although clinical application has already been performed. In this project MDA amplification from the minimum amount of DNA available was assessed using two multiplex PCR reactions that have already been applied in clinical PGD (Kakourou et al., 2007; Moutou et al., 2004). The reason for choosing well established and efficient PCR reactions was to minimise the ADO and TAF events due to PCR inefficiency and concentrate on the potential of MDA. In most of the related work that has been done more than six PCR markers were analysed from each MDA sample. However, in this project it was considered that using two well designed PCR reactions could provide realistic and accurate conclusions.

Since the MDA reaction was first described by Lizardi et al. (1998) for circular DNA and later by Dean et al. (2002) for linear DNA it was tested in various DNA samples with different DNA quantity and quality. When the starting DNA template was 10ng or greater, the results obtained were very promising, with more than 99% genome coverage (Dean et al., 2002; Paez et al., 2004). Even direct MDA on blood spots could give results similar to gDNA (Dean et al., 2003). Apart from genome coverage clear advantages over the PCR-based WGA methods are the large DNA strands that are synthesized, the final DNA yield and the low amplification bias. The results from this project show that MDA of gDNA samples were 100% accurate and followed similar stutter peak patterns compared to direct gDNA. Sometimes the stutter peaks however could be higher than expected and have a similar peak height to the real peaks (figure 3.4). This occurrence, which was not consistent in all cases, probably occurred due to the high concentration of the PCR product on the ABI 310 genetic analyser rather being an MDA artefact.

Testing at the single cell level is a much more challenging project as only two copies of the DNA template are available and new parameters, such as MDA-AE and ADO complicate the results. The first projects that concentrated on single cell MDA were published in 2004 (Hellani et al., 2004; Handyside et al., 2004).

Handyside published his results on five and eleven MDA products from single lymphocytes and single blastomeres respectively. The results were based on a very limited number of MDA products. MDA products from lymphocytes gave 100% MDA-AE, 31% ADO, 8% TAF and 2% inaccurate alleles whereas MDA products from blastomeres showed an overall 91% MDA-AE and 16% ADO.

Hellani et al. (2004) published the most controversial data on PCR with MDA samples from single cells. These results were based on 40 single lymphocytes that were lysed with proteinase K prior to MDA and digested and purified prior to PCR on 16 STR markers and a known mutation of the  $\beta$ -globin gene. According to his findings, MDA-AE was 97.5%, TAF was 0.0% and overall ADO was 1.9% (10.3% for the  $\beta$ -globin gene, 5.0% for D2S1338 STR marker and 0.0% for the rest 10 heterozygous STR markers). Our

results are in complete contrast with Hellani's findings since when using proteinase K lysis prior to MDA only 23.3% of the results gave the expected allele size for lymphocytes. A year later Hellani's group published the first clinical application of MDA in PGD (Hellani et al., 2005). Single cells this time were lysed using ALB lysis and not proteinase K, MDA-AE was 97.8%, average ADO was 14.1% and TAF was 0.0%. For 10 blastomeres that were used in two clinical PGD cases for thalassaemia and cystic fibrosis, MDA-AE was 80.0% but ADO and TAF were absent.

Since then a number of reports on single cell MDA and molecular analysis have been published even though it is a very specialized topic. Among them, the most important publications were from two groups, one from Belgium (Spits et al., 2006) that presented a detailed analysis from MDA products from single lymphocytes and one from the UK (Renwick 2006; Renwick et al., 2007) that evaluated and developed PGH. Renwick's group could not be directly compared to our results since they use a completely different definition for ADO in their results. They consider ADO to be calculated as "the number of alleles that failed to amplify from the total number of expected alleles at the heterozygous loci and TAF was counted as two ADO events" whereas in our results and every other groups, ADO is considered as the random non amplification of one of the alleles present in a heterozygous sample (Ray and Handyside, 1996). Although TAF and ADO could have a similar nature of occurrence they should not be combined together since ADO can only be detected in heterozygous samples whereas TAF can be detected in all samples. By modifying Renwick's calculations according to the analysis method that was used in this project for buccal cells an overall 38.8% ADO and 4.1% TAF were determined compared to 27.0% ADO that the group claimed. For blastomeres the EAS was 64.8% according to their results.

Results from other groups are compared in detail with our results in the following pages (Glentis et al., 2009). Unfortunately, most of the other published work was mainly reporting application of MDA on clinical PGD.

### 3.7.1 Cell lysis

Cell lysis is considered a crucial step for amplification by MDA as it enables exposure of the DNA making it accessible to the Phi29 polymerase. For this reason specialized protocols on single cell lysis were preferred over the standard cell lysis protocol from the Repli-G kit (Glentis et al., 2006b). From the two widely known lysis methods (alkaline and enzymatic), MDA produced much better results when the cells were lysed with ALB. Interestingly, the only research project that presented results for MDA single cells following proteinase K lysis was in 2004 (Hellani et al., 2004). Proteinase K lysis showed slightly better results in buccal cells compared to lymphocytes prior to PCR irrespective of MDA application (tables 3.4 and 3.5). This could be explained by the different nature of the cells including their morphology and DNA compaction. It should be noted that proteinase K has been extensively used by our laboratory and others on single buccal cells prior to application of DOP-PCR in research and clinical PGD m-CGH analysis (Mantzouratou et al., 2008; Obradors et al., 2008; Wells et al., 2002). It seems that the lysis of a cell should first depend on the downstream application that will be followed and secondly on the type of cell.

Two ALB lysis buffers (L1 and L2) were used with the only difference being the absence of DTT in L1. The two lysis buffers were compared in order to test if any variation would be detected from the results. Previous results from the Mayo clinic in the USA on MDA products from single fibroblasts showed excellent results when DTT was excluded from the lysis buffer (Schowalter et al., 2007). The same MDA products from single fibroblasts and blastomeres were sent to our laboratory from the Mayo group in 2007 and analysis of the two triplex PCR showed 100% accurate results with no ADO and TAF present (results not shown). Interestingly, the stutter pattern was similar to gDNA and no background artefacts were noticed. DTT has been shown to be necessary in single sperm studies as it reduces protamines by destroying disulphide bonds thereby allowing tightly packed DNA to become accessible to PCR primers. However, DTT may not be necessary for lysis of other cell types and its residual form may reduce PCR efficiency (Pierce et al., 2002; Cui et al., 1989). Spits et al (2006) compared two lysis buffers which contained

50µm and 100µm DTT and concluded that there was not a significant difference between the two buffers although a small difference was noticed favouring experiments with 50mM DTT. Schowalter et al (2007) concluded that DTT was not necessary for single cell MDA. Our experiments on the MDA reaction using a lysis buffer that contained DTT (50mM) on single buccal cells and lymphocytes showed a decreased MDA efficiency and accuracy on buccal cells and no difference on lymphocytes compared to cells that were lysed without DTT. In all the relative literature published DTT is used in 50µM or 100µM concentrations and volumes of 0.5µl to 2.5µl. It has been commented by other groups that ADO variation depends primarily on the cell type and the region of DNA that is amplified (Hellani et al., 2008; Burlet et al., 2006). Our results show that lysis also plays a crucial role in the ADO variation.

### **3.7.2 Cell types**

Different cell types have been described previously for PGD workups and other studies at the single cell level (Renwick et al., 2007; Fiegler et al., 2007; Spits et al., 2006). There are crucial differences between the cell types that are chosen. Buccal cells are easy to obtain for genetic testing but their DNA is degenerate as these cells are already dead or they have started the process of apoptosis (Piyamongkol et al., 2003). Lymphocytes are very small cells (7-9µm diameter) which make the isolation more difficult compared to other cell types. Their nucleus is large and it encompasses most of the cell, the cytoplasm is tiny and the DNA is relatively dense (Loiko et al., 2006). The fibroblasts that were collected for this project were actively dividing cells isolated from culture flasks before confluence. The DNA may be similar to a blastomere or a carcinogenic cell that may be dividing and therefore may have double the amount of DNA template available for the MDA reaction. The blastomeres used for this study were obtained from embryos that were excluded from embryo transfer which make them very likely to have chromosomal abnormalities. The majority of PGD workups are done on lymphocytes (Hellani et al., 2005; Lledo et al., 2007) and fewer groups have reported workups on buccal cells (Obradors et al., 2008; Renwick et al., 2006). Our results show a clear benefit of lymphocytes and fibroblasts over buccal cells that can be explained by the superiority of

DNA quality from these cell types. Blastomeres have comparable results with lymphocytes and fibroblasts but only when 10/34 cells that did not amplify at any locus were excluded. For two markers (APOC2 and D19S112) the ADO was relatively low (6.3% and 5.5% respectively) in contrast with the DM locus from which a 20.8% ADO rate was obtained.

### **3.7.3 MDA-AE**

A marked difference in terms of MDA-AE was observed between the MDA products from single buccal cells (82.0%) compared with lymphocytes and fibroblasts (96.0% and 100% respectively). MDA-AE for lymphocytes and fibroblasts in this study correlated with other studies (Lledo et al., 2007; Ren et al., 2007; Burlet et al., 2006;) but MDA-AE for buccal cells yielded 100% in one publication (Obradors et al., 2008) and was not mentioned in another (Renwick et al., 2006). Since MDA-AE has been calculated as the number of MDA products from single cells that would give at least one result for the tested loci, it is clear that the more loci that are used, the higher would be the MDA-AE. However, it was our belief that if MDA would fail to amplify all six markers it would be enough to conclude that the amplification efficiency was so low that it was similar to MDA failure. Likewise, for MDA application on single blastomeres, MDA-AE was not calculated because three markers located on the same chromosome were not considered enough to give a reliable result. As the blastomeres were obtained from embryos of PGS cases the chance of a chromosomal abnormality involving chromosome 19 was possible. It should be noted that MDA-AE could not be tested by measuring DNA concentration since DNA measurements show DNA existence even in negative results (Renwick et al., 2007; Spits et al., 2006). It is believed that MDA failure is mainly due to DNA quality of the single cell as it has been previously reported from various studies on gDNA samples of low molecular weight (Barber et al., 2006; Montgomery et al., 2005).

### 3.7.4 TAF and ADO

The biggest difference observed on MDA products from buccal cells compared to lymphocytes and fibroblasts was TAF. An average of 20.8% TAF was obtained from buccal cells compared to 6.6%, 7.5% and 6.9% from lymphocytes, fibroblasts and blastomeres respectively indicating that buccal cells may not be a suitable source of DNA to facilitate reliable MDA amplification. DNA degradation could explain the TAF difference between buccal cells and the other cell types studied. There is a large variation of TAF among different published results. Hellani et al. (2004, 2005, 2008) and Ren et al. (2007) indicated very low TAF for lymphocytes and blastomeres (zero up to 5%), Burlet et al (2006) showed a 5-15% and 26-39% TAF for lymphocytes and blastomeres respectively and Spits et al (2006) published an average of 9.2% TAF (61/600) on MDA products from single lymphocytes. In our blastomere results, if the ten excluded cells were included in the analysis, the average TAF would increase to 34.2% from 6.9%. However, these cells were excluded from the analysis because it could not be determined if the alleles did not amplify due to MDA failure or due to actual TAF of the PCR post-MDA. Renwick et al. (2006 and 2007) did not include TAF in their results but rather considered TAF as two ADO events. By analysing their data on MDA products from buccal cells with our parameters an overall 4.1% (3/72) of TAF was observed in the heterozygous loci (no information was given for the homozygous loci and so could not be included in the analysis). Obradors et al. (2008) did not detect any TAF on MDA products from single buccal cells. The results from these two groups are in contrast with our results which consider TAF as the largest problem encountered with MDA on single buccal cells. It should be noted that even in direct PCR on single buccal cells TAF has been calculated to be higher than the results published from these groups (Table 3.3; Sumita et al., 2007; Fassihi et al., 2006). According to our results, high TAF was expected as DNA from buccal cells is considered to be degenerated and this could hamper MDA function.

In our results ADO was calculated to be 31.0% for buccal cells, 20.8% for lymphocytes and 20.0% for fibroblasts. MDA products from blastomeres had an average of 12.1%



ADO for the DM triplex. However, if the two inconclusive results were counted as ADO, blastomeres would have 15.6% ADO which would be very similar to the 17.5% and 18.0% ADO of lymphocytes and fibroblasts for the DM loci respectively. ADO is a phenomenon that has been associated primarily with limited DNA availability and less with poor quality of DNA as it is mainly abundant at the single cell level. The high inter-locus variation observed shows that ADO also depends on the region of amplification. For buccal cells, Renwick et al. (2006) reported an average of 27.0% ADO although according to the actual definition of ADO their average rate rises to 38.8% for the results they presented. Having a universal presentation of the results would be helpful for the comparison of similar research projects. Obradors et al. (2008) published an average ADO rate of 20%. Nevertheless, in the limited published papers regarding MDA and single buccal cells it seems that ADO varies greatly but is usually higher compared to MDA products from single lymphocytes.

Regarding MDA on single lymphocytes, many more groups have published their data but the inconsistency of the results is even greater. Hellani et al. (2004), Lledo et al. (2006) and Ren et al. (2008) presented very small ADO (zero to 9%), Burlet et al. (2006) had an average of 20% ADO, Spits et al. (2006) presented an average of 26% ADO and Handyside et al. (2004) published an average of 31% ADO from a small number of MDA products. Interestingly, the research groups that have published high ADO rates for lymphocytes experienced lower ADO rates on blastomeres (Glentis et al., 2009; Burlet et al., 2006) whereas the groups that show small ADO rates on lymphocytes had increased ADO in their clinical PGD cases with MDA (Ren et al., 2008; Lledo et al., 2006; Hellani et al., 2005)

From the ADO results in 59.2% (58/98) of the total cases the larger allele was lost. However, considering heterozygous alleles that were more than 2bp apart, ADO of the larger allele occurred in 50.6% (40/81) of cases, indicating that ADO happened irrespective of allele size. In theory, ADO in MDA should happen irrespective of allele size as it is an isothermal amplification with effectively no upper limit on strand extension length. However, this finding could be explained by the different stutter bands

present in the MDA products. As stutter peaks were usually larger and different from the gDNA peaks for alleles that were very close together, ADO of the smaller allele may be present but “hidden” and confused with a stutter peak. Therefore in heterozygote samples that were 2bp apart, ADO was detected only in the larger allele (17/17). Similar concerns regarding confusion encountered from heterozygous alleles very close to each other have been reported previously following single cell MDA (Spits et al., 2006). According to this analysis it is very likely that some ADO events were confused with the stutter peaks in alleles with only 2bp difference due to the noise of the results.

Overall from our TAF and ADO results we conclude that (a) although MDA increased the total amount of DNA prior to PCR it showed higher rates of ADO compared to direct single cell PCR (Moutou et al., 2004; Piyamongkol et al., 2002) and (b) the main problem of MDA of single cells of low quality was TAF and not ADO. Thus good quality DNA was essential for robust MDA. The results from some groups that had completely different outcomes from ours regarding TAF and ADO (Hellani et al., 2008; Hellani et al 2004; Lledo et al., 2007; Lledo et al., 2006; Ren et al., 2007) give the impression that MDA should be considered as a favourable technique over direct PCR on single cells. In contrast, our results show that the advantage of MDA is that it produces large amounts of DNA that can be used for more genetic analysis and minimise the chance of misdiagnosis.

### **3.7.5 Inaccurate alleles, artefacts and bleed-through**

A total of four alleles (three from buccal and one from lymphocyte MDA products) of inaccurate size were detected (figures 3.7 and 3.8). At least one other study reported inaccurate allele size from PCR analysis after single cell MDA (Renwick et al., 2006) but most studies do not report the presence of incorrect alleles, confirming the accuracy of Phi29 polymerase (Dean et al., 2002). Contamination was excluded as an explanation of the presence of such alleles because it would likely account for inaccurate results on all three alleles of the triplex PCR. Although the data collected were very limited in order to make reliable conclusions it should be mentioned that MDA products from buccal cells

had three times more inaccurate allele sizes compared to lymphocytes. The main difference between the two cell types is the DNA quality which implies that this could play a role in the presence of inaccurate alleles. Another hypothetical explanation is the presence of chimeras that have been detected from DNA sequencing studies (Lasken and Stockwell, 2007).

Whether the background noise of a result could be misunderstood as an incorrect allele would need further investigation but depending on the results obtained artefacts and bleed-through events could easily be distinguished from the real peaks. Bleed-through could be minimised by diluting DNA from PCR product or use singleplex PCR (Sioban SenGupta, personal communication). The presence of background noise is not well understood but it is most likely that it begins due to traces of contaminating DNA or due to an undefined mechanism that does not require any template other than the primer population (Hutchison and Venter, 2006). Since the only available DNA comes from a single cell, even if a small amount of non-specific DNA is produced, it would be easily detected. Some groups have managed to minimise the background noise by minimising the reaction volume to 60-600nl (Marcy et al., 2007; Hutchison et al., 2005). However, that was technically impossible to do in our laboratory. The comparison of 25 and 40 PCR cycles showed that artefacts and bleed-through was diminished when fewer cycles were used but still existed. However, in some cases the real peaks were also underrepresented (figure 3.7B large allele) and could be misdiagnosed as ADO. It was concluded that less PCR cycles minimise background noise but also increase the chance of misdiagnosis.

### **3.7.6 Conclusion**

The results obtained from a large number of MDA products from single cells from various cell types gave several conclusions. MDA amplified successfully DNA from single cells, in the majority of cases, especially on cell types of good DNA quality. Inaccurate alleles were found only in four cases but ADO and TAF were more often present compared to single cell PCR. As single cell PCR showed better results compared

to PCR post MDA of single cells, the use of MDA for PGD should be selected for specific cases. The amount of DNA that is generated is the great advantage of MDA and could be helpful to minimise misdiagnosis by using direct and indirect diagnosis (Obradors et al., 2008). Work-up time for a specific genetic mutation could be minimised by using singleplex PCR. MDA could also be used for combination of molecular diagnosis with PGS (Obradors et al., 2008) or for the diagnosis of more than one genetic disorder. (Fiorentino et al., 2006). PGH can also be performed with MDA although from the results presented by Renwick et al. (2006 and 2007) a number of markers produced a very high ADO rate which means that PCR reactions for MDA should be well optimised.

The preclinical evaluation in a limited number of cells (Ren et al., 2007; Lledo et al., 2006) was considered to be very unreliable as high ADO and TAF could lead to misdiagnosis. According to our results, buccal cells were easy to obtain but were not favourable for MDA evaluation. The high variability of results presented from various groups demonstrates the need of more detailed research prior to the wide application of MDA to clinical PGD. The high background noise developed from the technique does not seem to influence PCR analysis but should be eliminated for other applications of MDA and single cells. More work should be done on single blastomeres for more reliable and accurate results. However, due to the difficulty of obtaining single blastomeres and in combination to the fact that too much work was done to optimise the reaction on other cell types it did not allow further experiments to be performed on single blastomeres. In conclusion, MDA can be used as a universal step prior to molecular analysis for PGD.

## **Chapter 4**

### **Analysis of single cells by a-CGH**

#### **4.1 Hypothesis**

The hypothesis of this chapter was that a-CGH can accurately assess the chromosomes of a single cell.

The difficulty of answering this hypothesis has mainly to do with the limited amount of DNA available and the sensitivity of a-CGH. As WGA of single cells is necessary for a-CGH experiments, uneven amplification of the genome together with artefacts and unspecific DNA formed by the amplification techniques would hamper the effectiveness and accuracy of a-CGH.

#### **4.2 Introduction**

Five years after the development of metaphase CGH (Kallioniemi et al., 1992), the first approach of a-CGH was published (Solinas-Toldo et al., 1997). Two basic reasons for this evolution were the higher resolution that could be achieved and the automation of the analysis. A-CGH has evolved very fast over recent years especially with the use of COT-1 DNA that blocks repetitive sequences (Craig et al., 1997), dye swap experiments and the resolution increase with the use of oligo or tiling path BAC arrays.

In PGS, aneuploidy screening of blastomeres is routinely performed by FISH (Baart et al., 2007) but m-CGH has been applied (Wilton et al., 2001; Wells et al., 2002; Obradors et al., 2008). Both techniques have limitations (sections 1.5.5 and 1.5.6). An automated technique that could provide information within two or three days could be very beneficial.

Very few studies have been reported regarding single cell a-CGH. The first approach was made in 2004 (Hu et al., 2004). Since then a limited number of groups have published their results (Hellani et al., 2004; Le Caignec et al., 2006; Feigler et al., 2007; Iwamoto et al., 2007; Fuhrmann et al., 2008). Each group used different array platforms (BAC arrays, SNP arrays, oligo-arrays) and WGA methods (MDA, GenomePlex, DOP-PCR, LA-PCR) whereas some groups have concentrated on optimising the WGA technique and others the array platform. The maximum resolution from single cell a-CGH has been claimed to be as high as 5MB (Fuhrmann et al., 2008) but in the first clinical PGS case that was reported recently whole chromosome changes were considered (Hellani et al., 2008).

### **4.3 Aims**

The aims of this chapter were to produce WGA (MDA and GenomePlex) products from various types of single cells (lymphocytes, fibroblasts and blastomeres) that can be used for accurate and reliable chromosomal analysis by a-CGH. Application of good quality gDNA on a-CGH can provide very high resolution analysis but at the single cell level high resolution analysis seems very difficult and challenging.

A-CGH could be very useful for the investigation of chromosomal abnormalities at the single cell level in different biological fields. PGS would benefit from analysis of all chromosomes from blastomeres before embryo transfer. A-CGH could be helpful for PGS as it allows diagnosis within 2-3 days so the need of cryopreservation would be eliminated. Further applications of a-CGH and single cells could be obtained for research purposes such as for the analysis of chromosomal imbalances in blastomeres. Similarly, the application of a-CGH in single tumour cells could give more detailed information about cancer mechanisms from the few cells that actually promote chromosomal imbalances in tumours.

#### 4.4 Experimental Design

For the purposes of this study, two different WGA techniques were applied on single cells and the WGA product was applied on BAC array slides from different companies or institutions. The cells were initially lysed with L1 lysis (ALB lysis without DTT, table 2.1) and amplified with MDA protocol P2. Alternatively, the cells were lysed with L1 or L3 lysis (proteinase K) and subjected to GenomePlex amplification (Section 2.4.2, table 2.2). Single lymphocytes, blastomeres and fibroblasts were used for this study as well as gDNA for positive controls. The WGA products were purified using a DNA clean-up kit and the DNA measured using a spectrophotometer. For MDA products, PCR analysis of the DM 1 locus was performed in order to confirm successful amplification. A-CGH slides from three companies (Spectral Genomics, Array Genomics and BlueGnome) and two institutions (Sanger Institute and Translational Research Laboratory, TRL) were used. All the slides were BAC arrays with various resolutions of 10MB (about 400 clones), 1MB (about 4000 clones) or tiling path (32000 clones). FISH experiments were performed in order to confirm the uniformity of the fibroblasts and to determine chromosome abnormalities in blastomeres.

The results were separated into four sets of experiments. Experiments A were performed with the Sanger Institute slides. The results are presented separately because except for the WGA method, the arrays were run at the Sanger centre by another person and information was provided (only graphs of the final results and not analysis data like percentage of clone inclusion and standard deviation for autosomal and sex chromosomes). Experiments B were performed with gDNA and MDA products from gDNA which were used as positive control experiments and to demonstrate which slide was providing the best results. Experiments C were conducted with WGA products from single lymphocytes and fibroblasts and finally experiments D were performed with WGA from single blastomeres. Figure 4.1 summarises all the main experiments. Apart from Sanger arrays, all a-CGH experiments were conducted by the author in the North Thames Regional Cytogenetic Laboratory, London.

## **4.5 Materials and Methods**

### **4.51 Ethical approval**

For blastomeres and embryos, Human Fertilisation and Embryology Authority (HFEA) approval was obtained together with written consent from the couples for donation of their embryos for research purposes.

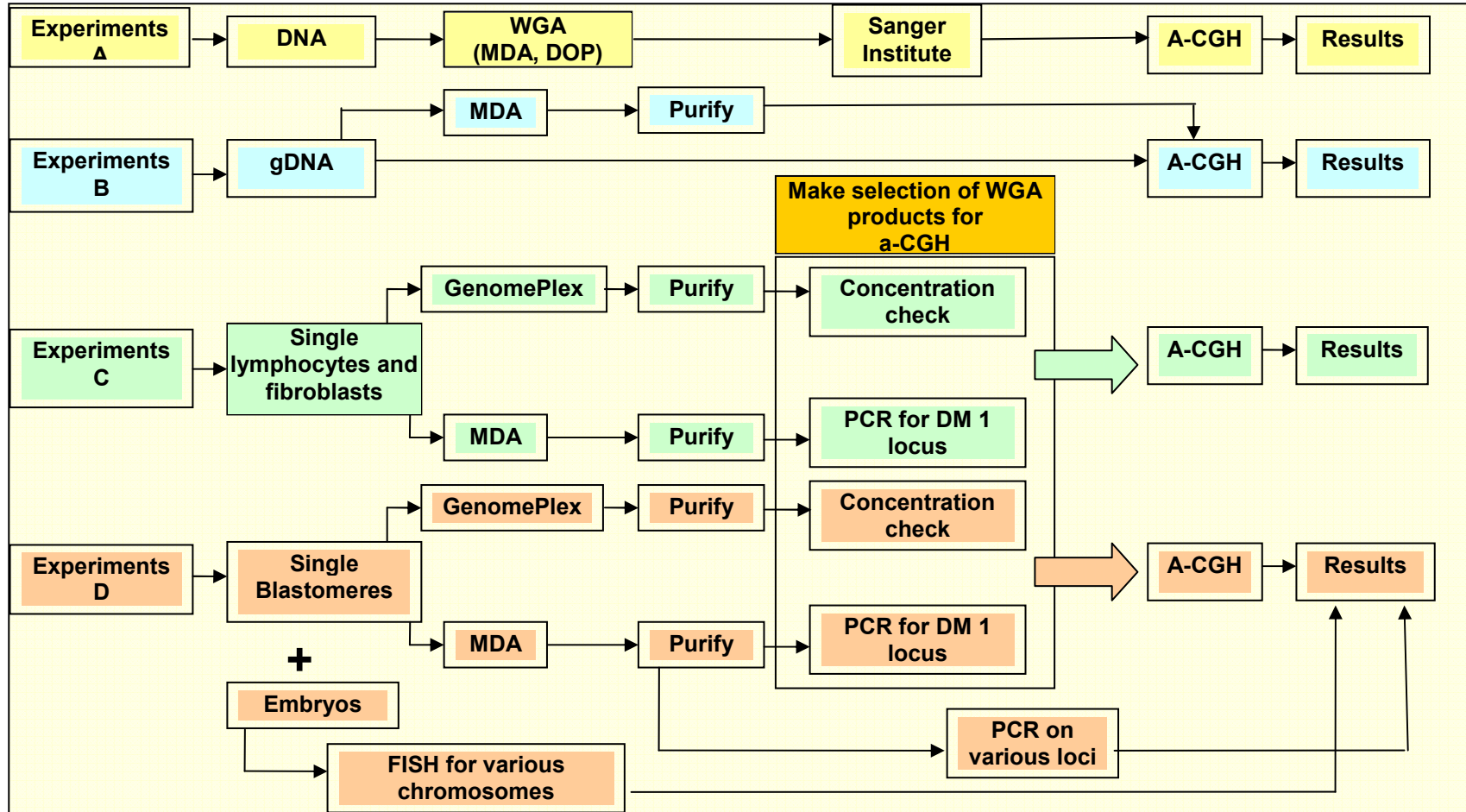
### **4.5.2 Materials and DNA samples for a-CGH**

Various DNA samples were used for single cell a-CGH experiments. Fourteen single lymphocytes, two single trisomic fibroblasts and five single blastomeres were subjected to WGA for the amplification of their DNA for molecular cytogenetic downstream reactions. MDA from control DNA as well as non-treated DNA with known chromosomal abnormalities was used for control experiments. A single cell DOP-PCR sample was provided by another student (Leoni Xanthopoulou) for one experiment. A total of 24 a-CGH experiments were conducted with slides from different companies and institutions (Sanger Institute, Spectral Genomics, Array Genomics, TRL and BlueGnome)

### **4.5.3 Cells and DNA treatment before a-CGH**

All lysis and WGA methods used for this chapter are described in sections 2.3 and 2.4. Single cells were lysed for DNA extraction prior to WGA. For MDA method L1 lysis (ALB lysis without DTT) was used. MDA protocol P2 (2 hours incubation) was used for the amplification of DNA from single cells whereas protocol P3 (protocol for gDNA) was used for DNA amplification of gDNA. For GenomePlex both L1 and L3 (proteinase K lysis) were used prior to amplification. The protocols that were used to amplify the





**Figure 4.1: Experimental design of the single cell a-CGH experiments.** This figure presents a summary of the main experiments that were conducted in this chapter. Four sets of experiments were performed which are shown in different colours in the diagram. DNA was first amplified by WGA and purified and then applied to a-CGH after selection through PCR and concentration measurement.

single cells were P6 (proteinase K lysis method) and P7 (alkaline L1 lysis) whereas gDNA was amplified with GenomePlex using protocol P5 (protocol for gDNA). All the DNA or WGA products were cleaned using Zymo research TM-25 clean up kit. After DNA purification the DNA concentrations were measured using a spectrophotometer ND-1000 and its computer program ND-1000 software. The optical density (OD, 260nm/280nm wavelength) was also scored for DNA purity. For pure DNA, OD should be 1.80. Numbers above 1.80 indicate the presence of RNA and numbers below 1.80 imply the contamination with proteins. In order to verify that MDA amplification of single cells had worked, all the single cell MDA products were subjected to PCR analysis for the DM I triplex (section 3.5.4.1). These markers were used only to determine that MDA correctly amplified the DNA from a single cell and the NanoDrop measurement was not due to unspecific DNA. The MDA products in which PCR had the most accurate genotypes were used for the a-CGH experiments. PCR was not applied to single cell GenomePlex products because the technique would not work due to the short length products that are produced (section 3.2). In two MDA products from single blastomeres, PCR was also applied for detection of specific chromosomes and gender determination. The markers used were located on chromosome 5 (markers D5S1965, D5S2065, D5S656 and D5S346), chromosome 7 (p.Phe508del and IVS8CA) and sex chromosomes (amelogenin gene). The PCR reaction for the markers on chromosome 7 is already reported (section 3.5.4.2). For markers on chromosome 5, the reactions were performed in singleplex. All the markers had the forward primer labelled with FAM and 0.3µM of primers were used for each reaction. The rest of the reagents for the reaction and the conditions of the PCR are identical with the DM I triplex. The markers on the amelogenin gene were used for gender determination. PCR on a female sample would produce only one product (104 bp) and on a male sample two products (104 bp and 117 bp) due to the presence of the gene in X and Y chromosomes (Hellani et al., 2009). The forward primer of the amelogenin marker was labeled with FAM and 0.3µM of primers were used for the reaction. All the other reagents used for PCR were identical to the DM I triplex (section 3.5.4.1) and the reaction conditions were identical to DM I triplex apart from the annealing temperature which was 59°C.

#### **4.5.4 A-CGH**

Various microarray slides from different companies and research institutions were used; Sanger Institute (Cambridge University), Spectral Genomics, Array Genomics, the Translational Research Laboratory (TRL) (UCL) and BlueGnome. For each slide the optimised protocol from the company/institution was applied.

##### **4.5.4.1 Sanger Institute**

The Sanger Institute developed BAC and PAC array slides for prenatal diagnosis (Rickman et al., 2005). The clones were previously amplified with DOP-PCR. Four slides were hybridised on gDNA and MDA products from gDNA and single cells. The DNA and MDA products were produced at UCL and sent to the Sanger Institute for hybridisation and analysis. The array slides (Rickman et al., 2005) and the protocol followed (Fiegler et al., 2003) have been previously published.

##### **4.5.4.2 Spectral Genomics**

The Constitutional Chip<sup>TM</sup> 2.0 developed by Spectral Genomics (now sold to Perkin Elmer, USA) is an a-CGH platform that contains 434 BAC clones that are specific to known constitutional syndromes, disorder-causing subtelomeric regions and trisomies. Reagents used for this project are listed in Appendix A7. The labelling and hybridisation protocol used was provided from the company: Briefly, 1µg of each DNA sample was diluted in 50µl of nuclease free H<sub>2</sub>O and sonicated for 1 minute at 100 amplitude. The DNA was purified and eluted in 54µl of sterile water using the Zymo Research's DNA clean and concentrator TM-5. Each sample was split into two tubes in order to label them with different dyes. Twenty µl of 2.5x random primer solution (Invitrogen) were added to each tube, the DNA was denatured at 100°C for 5 minutes and immediately cooled on ice for another 5 minutes. Two master mixes (one for each dye) were prepared which contained Spectral Labelling buffer 2.5µl X (4x number of experiments + 0.2), Cy3 dCTP or Cy5 dCTP 1.5µl X (4x number of experiments + 0.2) and Klenow fragment 1µl x (4 x

number of experiments + 0.2). Five microlitre of the master mix was mixed with the DNA and the samples were incubated at 37°C for 1 hour. The samples were denatured again at 100°C for 5 minutes and cooled on ice for another 5 minutes; 5µl of the same master mix was added and incubated at 37°C for another hour. Five µl of 0.5 M EDTA was added to each tube to stop the reaction. After labelling, the differentially labelled DNAs (test and reference) were mixed together and 45µl of Spectral Hybridisation Buffer I was added together with 6.45µl of 5M NaCl and 65µl of isopropanol in order to precipitate the DNA. The samples were incubated at room temperature for 20 minutes and centrifuged at full speed for 30 minutes. The supernatant was discarded, and the pellet was rinsed in 250µl of 70% ethanol. The samples were centrifuged for 3 minutes at full speed, the supernatant was discarded and the pellet was left to air dry for 5 minutes. The DNA was resuspended in 5µl of sterile water and incubated for 10 minutes at room temperature. After complete resuspension of the pellet, 12µl of Spectral Hybridisation Buffer II was added and the sample was incubated first at 72°C for 10 minutes and then at 37°C for 30 minutes. Finally, 15µl of the DNA solution was applied on each sub-array under a 22 x 22mm coverslip and the slide was incubated in a hybridisation at 37°C for 16 hours.

For post-hybridisation washes, the coverslips were removed from the slides by manually agitating the slides in 2xSSC/0.1% SDS and the slides were washed in the same solution for 15 minutes at 45°C. The slides were incubated in 2xSSC/50% formamide for 15 minutes at 45°C and then in 2xSSC/0.1% SDS for 30 minutes at 45°C. Finally the slides were washed in 0.2xSSC for 15 minutes at room temperature twice, and centrifuged at 170g for 3 minutes to dry.

#### **4.5.4.3 Array Genomics**

Array Genomics is a French company that produces pre- and postnatal arrays. Each slides contains 950 BACs printed in triplicate in two separate areas allowing dye-swap hybridisations on the same slide. The clones are printed directly onto a 3D microscopic slide, without denaturation of the DNA. The clones cover most known micro-deletion

syndromes, telomeric regions and has a median resolution of 10MB. Three arrays were provided by Array Genomics and the reagents were supplied from Invitrogen and GE healthcare (dyes Cy3/Cy5). The protocol used was identical to the Spectral Genomics protocol (section 4.5.4.2).

#### **4.5.4.4 Translational Research Laboratory**

The Translational Research Laboratory (TRL, Institute for Women's Health) developed an a-CGH platform from the 'Golden Path' BAC clone set generated by CHORI (USA) and Genome Sciences Centre (Canada) (Mermaid 32K array). The 30,388 BAC clones were derived from the Human RPCI-11 and -13 libraries and the 'CalTech' Human BAC Libraries (CIT-D). The clones were amplified using MDA (TempliPhi kit, GE Healthcare) and purified using the Millipore MultiScreen PCR384 filter plates before printing onto slides using a QArray<sup>2</sup> robot.

The Bioprime genomic labelling system was used to label the DNA. Briefly, 800ng of DNA or WGA product were used as a starting material and diluted in 21µl of dH<sub>2</sub>O. Twenty microlitres of random primers were added to the DNA and the samples were denatured at 95°C for 15 minutes. This was followed by placing the samples on ice for 15 minutes and then 5µl of dNTP/low-dCTP mix, 2µl of Cy3 or Cy5 and 1µl of klenow fragment were added to each sample. The samples were incubated at 37°C for 18 hours. After the reaction was finished 5µl of EDTA were added to stop the reaction and the tubes were stored at 4°C.

The samples were purified prior to the precipitation and hybridisation process using Autoseq G50 columns following a standard protocol. Briefly, the columns were first vortexed for 30 seconds in order to resuspend the resin in the column and then the bottom closure was removed. The screw cap was loosened by one quarter of a turn, the columns were placed in a 1.5ml conical screw cap tubes and centrifuged at 5000rpm for 1 minute. The conical tubes that collected the supernatant were discarded and the columns were placed in new clean conical tubes. The labelled DNA was placed in the centre of the

remaining resin ensuring that no product would be left in the walls of the tube. The columns were centrifuged for 2 minutes at 5000 rpm. The clean labelled DNA was collected in the conical tubes and the columns were discarded. The incorporation of the dyes in the DNA was measured with the ND-1000 spectrophotometer using 1.5µl of labelled DNA.

DNA precipitation was carried out by combining the reference and the test labelled DNA and adding 100µg of human COT-1 DNA, 30µl 3M NaOH pH5.2 and 825µl of absolute ethanol. The DNA was precipitated at -20°C for 2 hours. After the precipitation step the samples were centrifuged at full speed for 30 minutes at 4°C, the supernatant was removed and the pellet was dried in a heat block at 37°C for 15 minutes. Hybridisation solution (50% formamide, 2% SDS, 10% dextran sulphate and 4µg/µl yeast tRNA) was prewarmed to 37°C for a few minutes until it became clear and 85µl were applied to the dry DNA pellet. The samples were incubated at 37°C and vortexed every 5 minutes until the pellet was completely dissolved. Once the pellet was dissolved the samples were denatured at 70°C for 5 minutes and re-annealed for 1 hour at 37°C.

Ten millilitres of fresh pre-hybridisation solution (25% formamide, 5xSSC, 0.1%SDS and 0.05g of BSA) was prepared and heated at 42°C. The array was denatured on a heat block at 95°C for 2 minutes and 200µl of pre-hybridisation solution were placed on top of the array spots. This was covered with a 22x54mm coverslip and the array was incubated in a hybridisation chamber at 42°C for one hour. The slides were rinsed in 0.1xSSC and dried by centrifugation at 170g for 2 minutes.

The samples were applied on a 22x54mm coverslip avoiding any bubbles and the slides were carefully lowered onto the coverslip. The slides were incubated in a hybridisation chamber at 42°C for 16 hours. The post-hybridisation washes were identical to Spectral Genomics protocol (section 4.5.4.2).

#### 4.5.4.5 BlueGnome

Two types of BlueGnome BAC array slides were used. The Cytochip 1MB V2 is an array of 6,000 BAC clones with a median of 565kb resolution of the human genome. The Cytochip prenatal Beta is a constitutional slide with 500 BAC clones that covers most of the known genetic imbalanced syndromes. Both types had two hybridisation areas for dye swap experiments.

Fluorescent labelling of test and reference DNA took place using the BlueGnome fluorescent labelling kit (BlueGnome, UK). Briefly 4µl containing 400ng of DNA were placed in a 0.2ml thin-walled PCR tube. Cy3 and Cy5 labeling mixes were prepared as follows: Reaction buffer 10µl x (2 x number of experiments + 0.4), nuclease free H<sub>2</sub>O 19µl x (2 x number of experiments + 0.4), Primer solution 10µl x (2 x number of experiments + 0.4), dNTP mix 5µl x (2 x number of experiments + 0.4) and Cy3 dCTP 1µl x (2 x number of experiments + 0.4) or Cy5 dCTP 1µl X (2 x number of experiments + 0.4). Once the labelling mixes were prepared, 45µl were added to the DNA and the samples were denatured at 94°C for 5 minutes. Following denaturation the samples were immediately placed on ice for another 5 minutes. For the labelling reaction to take place 1µl of klenow enzyme was added to each tube and the samples were incubated in a thermal cycler for 18 hours at 37°C. Once the reaction had finished 5µl of EDTA was added to each sample to stop the reaction. The purification of the labelled DNA and the measuring of the dye incorporation were identical to the protocol followed by TRL slides (described in section 4.5.4.4).

For precipitation, the tube was inverted twice to mix and precipitated in the dark for 2 hours at -20°C. After precipitation of labelled DNA, the samples were centrifuged at full speed for 15 minutes, the supernatant was discarded and the pellet was washed in 250µl of 70% ethanol. The samples were centrifuged again and the supernatant was discarded. Any remaining droplets were removed with a pipette and the samples were left to air dry for 5 minutes. The tubes containing a purplish pellet were stored in the dark at -20°C until required.

In a separate tube, 75µl of herring sperm DNA, 62.5µl of COT-1 DNA, 14µl of 3M sodium acetate pH 5.2 and 350µl of absolute ethanol per microarray experiment were mixed together and precipitated for 2 hours at -20°C. This mixture was used for the formation of the hybridisation solution. Similar to the labelled DNA, after precipitation the samples were centrifuged at full speed for 15 minutes, the supernatant was discarded and the pellet was washed in 350µl of 70% ethanol. The samples were centrifuged again and the supernatant was discarded. Any remaining droplets were removed with a pipette and the samples were left to air dry for 5 minutes.

Once the pellet was dried, 52.5µl of hybridisation buffer (10% dextran sulphate) per experiment was added and the tube was incubated at 75°C in a hot block for 10 minutes. Ensuring that the pellet has completely dissolved, 25µl of the hybridisation solution were added to each labelled DNA pellet. The tubes were incubated for 10 minutes at 75°C and flicked every couple of minutes to ensure that the pellet was completely dissolved. Once the pellets were dissolved the samples were denatured for a further 10 minutes. The Cytochip slides were incubated in 100ml PBS solution for 5 minutes and dried by centrifugation at 1000 rpm for 3 minutes. Twenty two microlitres of the pre-hybed DNA solution was applied on each sub-array under a 22 x 22mm coverslip. Each array was placed in a hybridisation chamber and incubated at 37°C for 16 hours.

Formamide-free washes were performed to remove any unbound labelled DNA from the Cytochip slides. The coverslips were removed from the Cytochip slides by manually agitating the slides in 2xSSC/0.05% Tween20. The slides were washed in 400ml of 2xSSC/0.05% Tween20 at room temperature twice for 10 minutes each. A third wash of the same solution at 60°C for 5 minutes was performed and this was followed by a 1xSSC wash at 60°C for 5 minutes. The slides were washed in 0.1xSSC solution at 60°C for 5 minutes and finally in 0.1xSSC at room temperature for 5 minutes. The slides were dried by centrifugation at 170g for 3 minutes.



#### **4.5.4.6 Scanning and analysis**

The images were scanned using a ProScanArray HT Microarray Scanner (Perkin Elmer, Finland). The analysis of the images was performed with the use of the BlueFuse for microarrays software provided by BlueGnome for the Cytochip slides, the TRL slides and the Array Genomic slides. Analysis for Spectral Genomics was performed with the use of SpectralWare™ online software. The process order for all experiments was first normalization and then exclusion. For Cytochip arrays, the Cytochip normalization software was used which has been designed by BlueGnome to provide best results for the company slides. The exclusion of the clones was performed by Cytochip normalization protocol. The software does not allow any changes to be made in the post-processed results and combined results by fusion. The log2 threshold for amplification and deletion was set to +0.2999 and -0.2999 respectively. In a number of experiments, analysis was also performed with a different algorithm (prenatal algorithm) that is used to identify only whole chromosome changes. This algorithm is currently developed by BlueGnome but no further information was provided for the analysis method that is being performed.

For the TRL 32K Mermaid arrays the Lowess 2D normalization method was used and clones were excluded when the confidence was less than 0.5 or replicates with SD were greater than 0.5. For the analysis of the Spectral Genomics and Array Genomics arrays, block lowess normalization method was used. No further information was provided for the Spectral Genomics analysis. For Array Genomics clones were excluded when the confidence was less than 0.3 or replicates with SD were greater than 0.1 or dye-swap replicates with SD greater than 0.2.

#### **4.5.5 FISH experiments**

FISH experiments were used to examine chromosome abnormalities of human embryos from which blastomeres were used in the a-CGH analysis and to score the fibroblasts in order to confirm the homogeneity of trisomies 13 and 21. DNA probes were all provided from Vysis UK and FISH solutions were provided by Sigma UK (Appendix A8).

#### **4.5.5.1 Slide preparation for skin fibroblast and lymphocytes**

Lymphocytes were used as positive controls. Cell suspensions were diluted with fix solution (three volumes of methanol and one volume of glacial acetic acid). With the use of a Pasteur pipette three drops of the cell suspensions were dropped on a clean moist slide from ~30cm height in order to spread the nuclei. With the use of a diamond marker a circle was engraved on the underside of the slide where the nuclei were spread. The slide was left to dry for two minutes and the slide was flooded with fix for 10 seconds. The fix solution was poured off, the slide was dried and 70% of acetic acid was added for a further 10 seconds. The slide was dried again and passed through an ethanol series (70%, 90% and 100%) for 5 minutes each. The slides were stored at 4°C and were used within 30 days.

#### **4.5.5.2 Slide preparation for human embryos**

Prior to embryo spreading the slides were prepared as follows; they were first washed in methanol/HCl for a few seconds, left to dry, incubated in 10% poly-L-lysine solution for 5 minutes, dried at room temperature and stored at 4°C. A small circle was drawn on the underside of each slide with a diamond marker. A drop of spreading solution was placed on top of the circle. Another drop (of PBS) was placed in the outer corner of the slide. The embryos were transferred from the Petri dish to the PBS solution with the guidance of a dissecting microscope and with the use of a plastic capillary and aspiratory micromanipulation. The capillary was first primed with spreading solution and then transferred the embryo to the drop of spreading solution. The lysis of the embryo was closely observed under the dissecting microscope and fresh spreading solution was added until complete lysis of the cells was achieved and the nuclei could be clearly seen without permitting the drop to dry. Once lysis was completed, the slide was left to dry and incubated in PBS for 5 minutes and dehydrated through an ethanol series (70%, 90% and 100%) for 5 minutes each. The slides were stored for up to two weeks at room temperature.

#### 4.5.5.3 Slide pre-treatment

The slides that contained the cells were first incubated in 1N HCl and 10mg/ml pepsin solution (Sigma, UK) at 37°C for 20 minutes (embryos for 15 minutes) in order to make the DNA accessible to the probes. After the incubation, the slides were briefly washed once in double dH<sub>2</sub>O and once in PBS. The slides were fixed in 1% paraformaldehyde (Sigma, UK)/PBS for 10 minutes at 4°C. After fixation, the slides were washed briefly once in PBS and twice in double dH<sub>2</sub>O and dehydrated through an ethanol series (70%, 90% and 100%) for 5 minutes each (Harper et al., 1994).

#### 4.5.5.4 Probe preparation

The probe mix consisted of probes for up to three chromosomes and buffer (CEP buffer for centromeric probes and LSI for locus specific probes). The total volume was always 5µl per sample. Table 4.1 summarises all volumes for probe mixes.

Probe mix	Amount	Buffer
13(SG)/21(SR)	13/21:0.6µl	LSI: 4.4µl
X(SG)/Y(SR)/18(SO)	2.5µl	CEP: 2.5µl
13(SG)/21(SR)/15(SO)	13/21:0.6µl/ 15:0.5µl	LSI: 2.5µl
1(SG)/ 5(SR)/19(SO)	1:0.6µl / 5:0.6µl / 19:0.6µl	LSI: 3.2µl

**Table 4.1: Summary of probe mixes used for FISH on fibroblasts and embryos.** A single round was used for the fibroblasts (chromosomes 13 and 21) and three rounds were used with each embryo. SG: spectrum green, SR: spectrum red and SO: spectrum orange.

#### **4.5.5.5 Separate denaturation, hybridisation and post-hybridisation washes**

In case of separate hybridisation, 100µl of denaturation mix (70% formamide/2xSSC) was added to the slides, covered with 22x50 coverslips and incubated at 75°C for 5 minutes. The coverslips were removed and the slides were dehydrated in 70% ice cold ethanol for 5 minutes and then in 90% and 100% ethanol at room temperature for 5 minutes each. The probe mix was denatured at 75°C for 5 minutes and stored at 37°C until the hybridisation step. Once the slides had dried, 5µl of probe mix was applied on the slide under a 13mm coverslip in the area where the circle was drawn. Rubber cement was applied around the coverslip and the slides were hybridised in a moist chamber at 37°C overnight.

Once the hybridisation was complete, the coverslip and the rubber cement were gently removed and the slides were washed in the dark three times in 50% formamide/2xSSC at 41°C for 5 minutes each and then 3 times in 2xSSC at 41°C for 5 minutes each. A 4xSSC/0.05% Tween20 wash for 5 minutes followed and a final dehydration series (70%, 90% and 100%) for 3 minutes each in the dark. After the slides were dried they were mounted in Vectorshield antifade medium counterstain (1.25ng/ml 4', 6-diaminidino 2 phenylindole (DAPI)) and stored in the dark at 4°C.

#### **4.5.5.6 Co-denaturation, hybridisation and post-hybridisation washes**

In co-denaturation, the probe mix was applied on the slide under a 13mm coverslip, denatured for 5 minutes at 75°C and hybridised for 1 hour without the use of rubber cement. After hybridisation the coverslips were gently removed and the slides were washed in 2xSSC/60% formamide at 41°C for 5 minutes, followed by 2xSSC at 41°C for 5 minutes and finally with 4xSSC/0.05% Tween20 for 5 minutes at room temperature. The slides underwent a final dehydration series and were mounted in Vectorshield antifade medium counterstain and stored in the dark at 4°C.

#### **4.5.5.7 Image analysis**

Fluorescent microscopy was achieved with the use of a Zeiss Axioskop microscope with chroma multi-band pass TRITC/FICT/DAPI filter and single Spectrum-Aqua filter. The same microscope with a built in Photometrics KAF 1400 cooled CCD (charged coupled device) camera was used to capture FISH images. SmartCapture software from Vysis was used to control the image capturing. The analysis was achieved using computer software (Digital Scientific, UK), that converted fluorescent intensities into a red, green or aqua colour for each signal.

#### **4.5.5.8 Re-probing of embryos**

In case of re-probing, after analysis of the slides was completed, the coverslips were removed and the slides were washed at room temperature in 4xSSC/0.05% Tween20 twice for 5 minutes each and then in PBS for 10 minutes followed by dehydration through an ethanol series. The slides were exposed to light during these washes in order for the old probe to fade. After dehydration the same slide could be used again.

### **4.6 Results**

#### **4.6.1 DNA and WGA product concentration and quality validation**

Concentrations of all the DNA and WGA products prepared for the array experiments are presented in table 4.2. PCR on the MDA products certified that the reaction was successfully performed (table 4.3). The presence of DNA of the MDA products was not detected by running an agarose gel because even negative MDA samples produce unspecific DNA that cannot be clearly distinguished from other MDA products. An example of how single cell MDA products appear on agarose gels and how they are compared with gDNA and gDNA-MDA products is illustrated on Appendix A9, figure A1. For lymphocytes and fibroblasts the true alleles were already obtained from chapter 3 (table 3.5) and so the MDA products that were used for the a-CGH experiments were the

ones that had the most accurate genotype. For blastomeres the expected alleles were unknown and so the MDA reactions' performance was based on the presence of alleles. In one case of a single blastomere MDA product, three alleles were detected in two loci (DM1 and APOC2) and two alleles on the third locus (D19S112) which implied either contamination or trisomy 19 (table 4.3). Although MDA incubation time of Phi29 polymerase was decreased to two hours in this project (protocol P2) from six (protocol P1) no difference was observed in the PCR results. For GenomePlex products, molecular analysis was not performed as the presence or absence of alleles would only depend on where the DNA was cut at the first step of the reaction. Measuring the concentration of the GenomePlex products was the only validation for the amplification efficiency of GenomePlex.

DNA template	WGA method - protocol	DNA conc. (ng/μl)	OD
gDNA male normal (Promega)	No WGA	220	1.8
	MDA P3	497	1.85
gDNA female normal (Promega)	No WGA	190	1.8
	MDA P3	363	1.86
gDNA 46, XY der(3)+ (3;15)(p24.3;q13)	No WGA	121	1.81
gDNA from cultured fibroblast T13	No WGA	67	1.78
	MDA P3	171	1.87
gDNA from cultured fibroblast T21	No WGA	89	1.77
	MDA P3	258	1.86
Single Fibroblast T21	MDA P2	60	1.86
Single Fibroblast T13	MDA P2	190	1.83
Single lymphocyte male 1	MDA P2	67	1.74
Single lymphocyte male 2	MDA P2	40	1.83
Single lymphocyte male 3	MDA P2	180	1.86
Single lymphocyte male 4	GenomePlex P6	11	1.42
Single lymphocyte male 5	GenomePlex P6	17	1.62
Single lymphocyte male 6	GenomePlex P6	146	1.77
Single lymphocyte male 7	GenomePlex P7	224	1.85
Single lymphocyte female 1	MDA P2	123	1.81
Single lymphocyte female 2	MDA P2	33	1.54
Single lymphocyte female 3	MDA P2	73	1.83
Single lymphocyte female 4	GenomePlex P6	299	1.85
Single lymphocyte female 5	GenomePlex P6	155	1.82
Single lymphocyte female 6	GenomePlex P7	283	1.79
Single lymphocyte female 7	GenomePlex P7	231	1.86
Single blastomere 1	MDA P2	166	1.84
Single blastomere 2	MDA P2	251	1.86
Single blastomere 3	GenomePlex P7	43	1.83
Single blastomere 4	MDA P2	234	1.79
Single blastomere 5	GenomePlex P7	6	1.46

**Table 4.2: DNA samples prepared for the array experiments.** The DNA concentration and the OD are displayed. Some samples with low DNA concentration were excluded from the experiments. Conc.: Concentration, OD: optical density.

MDA products/Markers	APOC2	DM1	D19S112
Single Fibroblast T13	127 150	122	139 141
Single Fibroblast T21	135 150	145 172	132 ADO
Single lymphocyte male 1	152 154	140 155	123 132
Single lymphocyte male 2	152 ADO	ADO 155	123 132
Single lymphocyte male 3	152 154	140 155	123 132
Single lymphocyte female 1	127 150	122	ADO 134
Single lymphocyte female 2	127 150	TAF	117 ADO
Single lymphocyte female 3	127 150	122	117 134
Single blastomere 1	152	122	123 141
Single blastomere 2	150	128 146	117
Single blastomere 4	135 150 154	128 146 181	117 130

**Table 4.3: PCR validation of MDA reaction with PCR.** This table presents the PCR results of the DM 1 triplex reaction of MDA products from single cells before a-CGH. MDA products from single lymphocyte male 2, and single lymphocytes female 1 and 2 were excluded from a-CGH experiments because ADO and TAF were present.

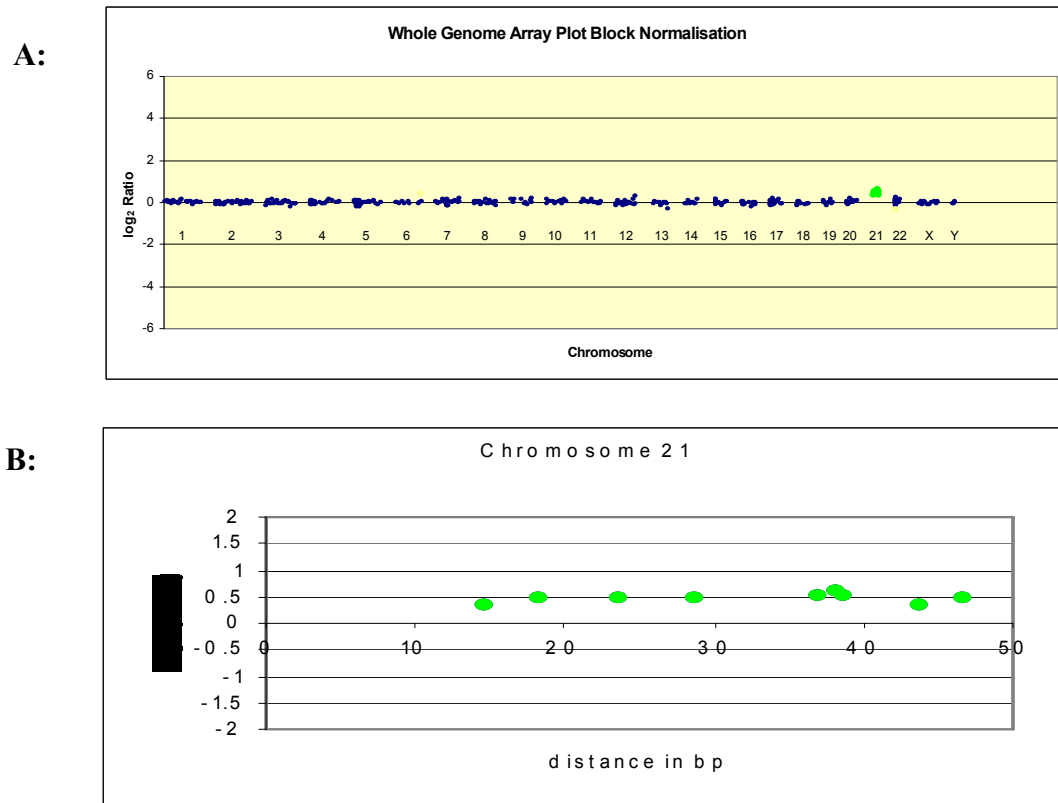
#### 4.6.2 Results from Sanger Institute a-CGH slides

The first a-CGH experiments that were performed for this project were from collaboration with the Sanger Institute. Four a-CGH experiments were conducted. The test and reference samples used for a-CGH are shown in table 4.4. The results obtained from these experiments were not promising. The only robust result obtained was from gDNA and inconclusive results were presented from all WGA products, even when MDA was applied using gDNA. The DOP-PCR amplified single cell that was hybridised to the Sanger Institute arrays was also used in a different project regarding single cell m-CGH and the result confirmed a trisomy 21 (figure 4.2).



Experiment number	Test sample	Amplification method	Reference sample	Result
A1	Fibroblast gDNA 47,XX+21	No amplification	Normal male gDNA	47,XY+21
A2	Fibroblast gDNA 47,XX+21	MDA, P3		Inconclusive
A3	Single Fibroblast T21	MDA, P2		Inconclusive
A4	Single Fibroblast T21	DOP-PCR		Inconclusive

**Table 4.4: Sanger Institute *a*-CGH experiments.** This tables shows the test and reference DNA used for each experiment as well as the result from the analysis.



**Figure 4.2: Graphs of the result from experiment A1.** The diagrams present the result of experiment A1, where a trisomy 21 was detected from gDNA. (A): Results from all chromosomes, (B): detail analysis on chromosome 21. Only clones on chromosome 21 show an increase of the  $\log_2$  channel1/channel2 ratio and the spots are highlighted in green.

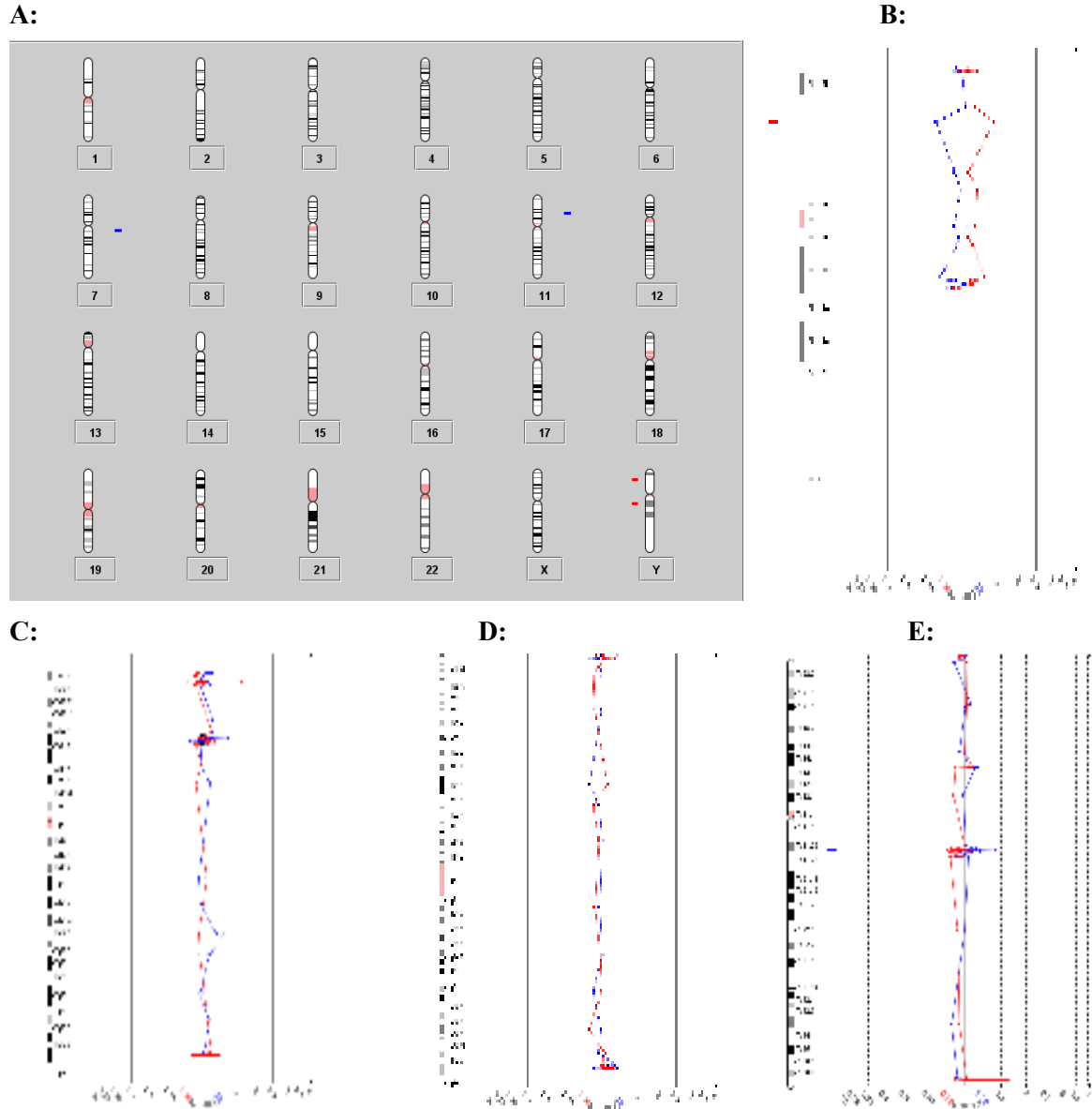
#### 4.6.3 A-CGH results with gDNA and MDA products from gDNA

Prior to single cell a-CGH, experiments using gDNA and MDA products from gDNA were performed in order to assess the efficiency and the quality of different slides. Table 4.5 shows all the experiments made with the different a-CGH slides together with the test and reference samples used in each case.

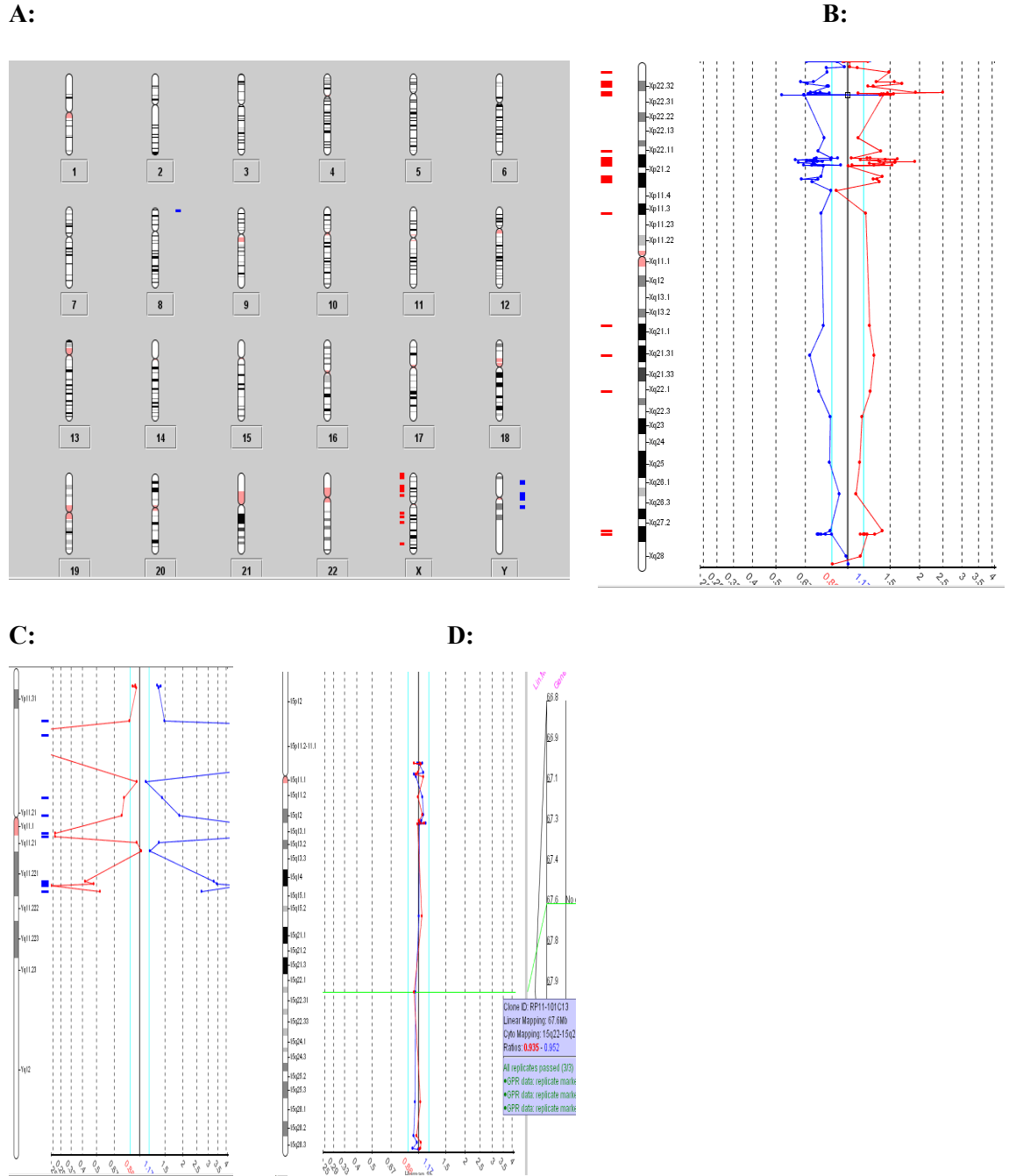
Exper. No	Array slide Company	Test DNA (gDNA)	Reference DNA (gDNA)	Spots analysed	Result
B1	Spectral Genomics 2.0 <sup>TM</sup>	Normal male	Normal female	93.69 %	Y chr. amplif.
B2	Spectral Genomics 3.0 <sup>TM</sup>	46, XY der(3) + (3;15)(p24.3;q13)	Normal female	96.58 %	Sex mismatch
B3	Array Genomics prenatal	Normal male	Normal female	87.34 %	Y chr. amplif.
B4	Array Genomics prenatal	Trisomy 13 male	Trisomy 21 female	56.65 %	21,X chrom. del. /13 amplif.
B5	Mermaid 32K (TRL)	Normal male	Normal female	78.26 %	Sex mismatch
B6	Cytochip prenatal (BlueGnome)	46, XY der(4) + (4;5)(q35.1;p14.3)	Normal female	99.85 %	Sex mismatch, chr. 5 amplif.
B7	Cytochip 1MB (BlueGnome)	MDA P3 Normal male	MDA P3 Normal female	99.81 %	Sex mismatch

**Table 4.5: A-CGH experiments with gDNA and WGA products from gDNA.** This table presents the a-CGH experiments performed with gDNA, indicating the source of the array slides, the test and reference DNA, the number of clones analysed and the result obtained from the array. Chr.: chromosome, Amplif.: amplification.

The Spectral Genomics constitutional chips 2.0<sup>TM</sup> slide failed to show a clear sex mismatch even with gDNA samples and only Y chromosome amplification was detected (figure 4.3). An upgrade version of Spectral Genomics 2.0<sup>TM</sup> slide (Spectral Genomics 3.0<sup>TM</sup>), was also tested by using an abnormal gDNA as test sample (46, XY der(3) + (3;15)(p24.3;q13)) and a normal female as a reference sample. Although a clear sex mismatch was detected, this experiment failed to show any chromosomal changes on chromosomes 3 and 15 (figure 4.4).



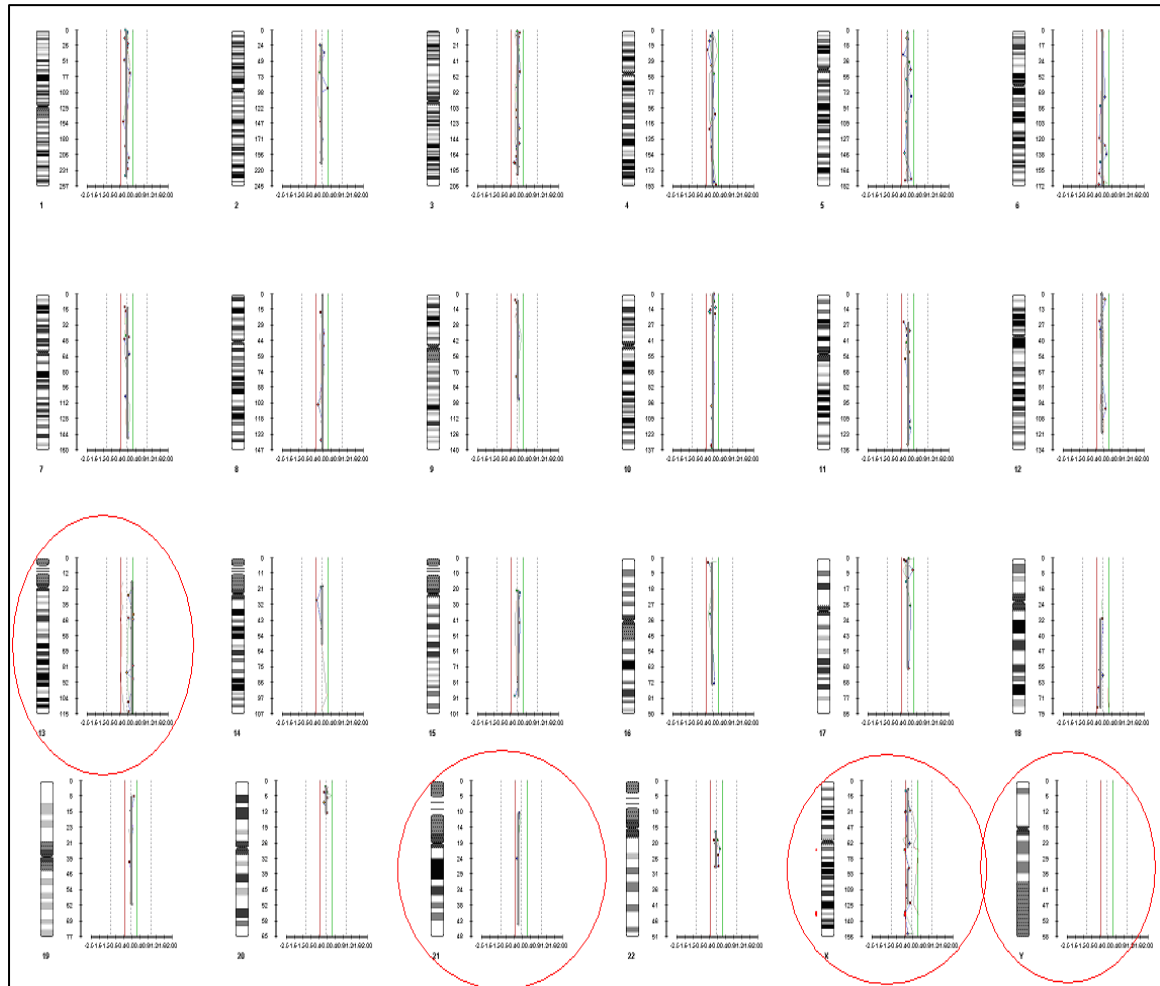
**Figure 4.3: Spectral Genomics 2.0™ experiment B1 Ideograms.** The figure shows the ideograms from all chromosomes (A) and detail on chromosomes Y (B), X (C), 1 (D) and 7 (E) after analysis of a-CGH images with SpectralWare™. No chromosomal imbalance was detected on chromosome X despite the 2:1 ratio in the test and reference DNA. The image for the X chromosome is similar to chromosomes 1 and 7 where the ratio was 1:1. A small deletion is seen on chromosome Y but only 2/12 clones exceed the threshold level.



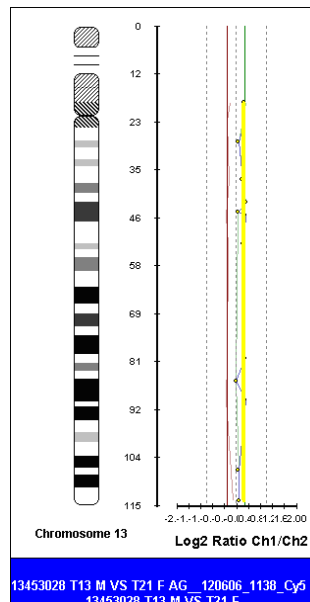
**Figure 4.4: Spectral Genomics 3.0™ experiment B2 Ideograms.** A clear sex mismatch was observed from the analysis of this experiment (A: Graph for all chromosomes, B: chromosome X, C: chromosome Y). However, no chromosome imbalance was detected for chromosomes 3 and 15 (D).

Two slides were used from Array Genomics. In the first experiment, normal male was hybridised against normal female gDNA. Similarly to Spectral Genomics, the Y chromosome showed a clear amplification but the X chromosome did not exceed the log<sub>2</sub> ratio threshold for deletion. In the second experiment, gDNA extracted from the embryonic fibroblast cell lines was used. A male sample with trisomy 13 (47, XY +13) was hybridised against a female sample with trisomy 21 (47, XX +21). Four chromosomal imbalances were expected to be scored. The analysis showed amplification on chromosome 13 and deletion on chromosomes 21 and X although they were not strong enough to exceed the desired log<sub>2</sub> intensity ratio threshold for imbalance. None of the clones representing the Y chromosome were included in the analysis so no conclusion could be made for that chromosome. Generally, the experiment had low clone representation (56.65%) (figure 4.5). In order to exclude mosaicism as a cause of low log<sub>2</sub> Ch1/Ch2 ratio for chromosomes 13 and 21, FISH experiments were done on the embryonic fibroblast cells for these chromosomes which confirmed the uniformity of the fibroblast cells (figure 4.6).

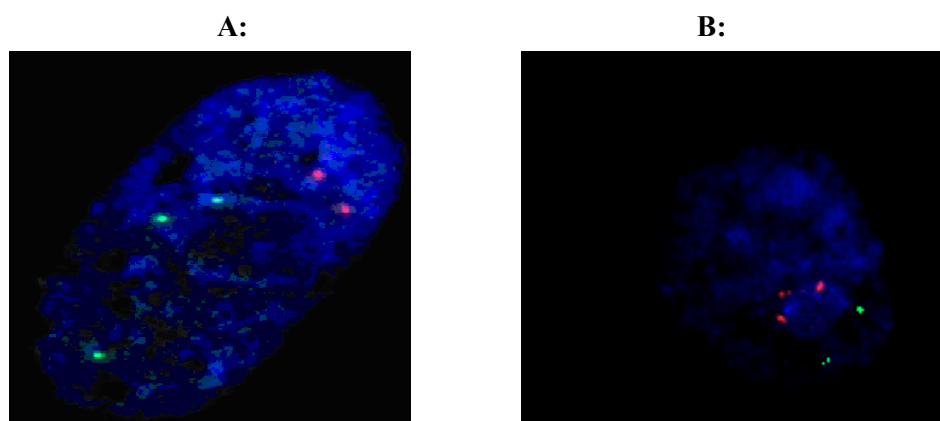
**A:**



**B:**

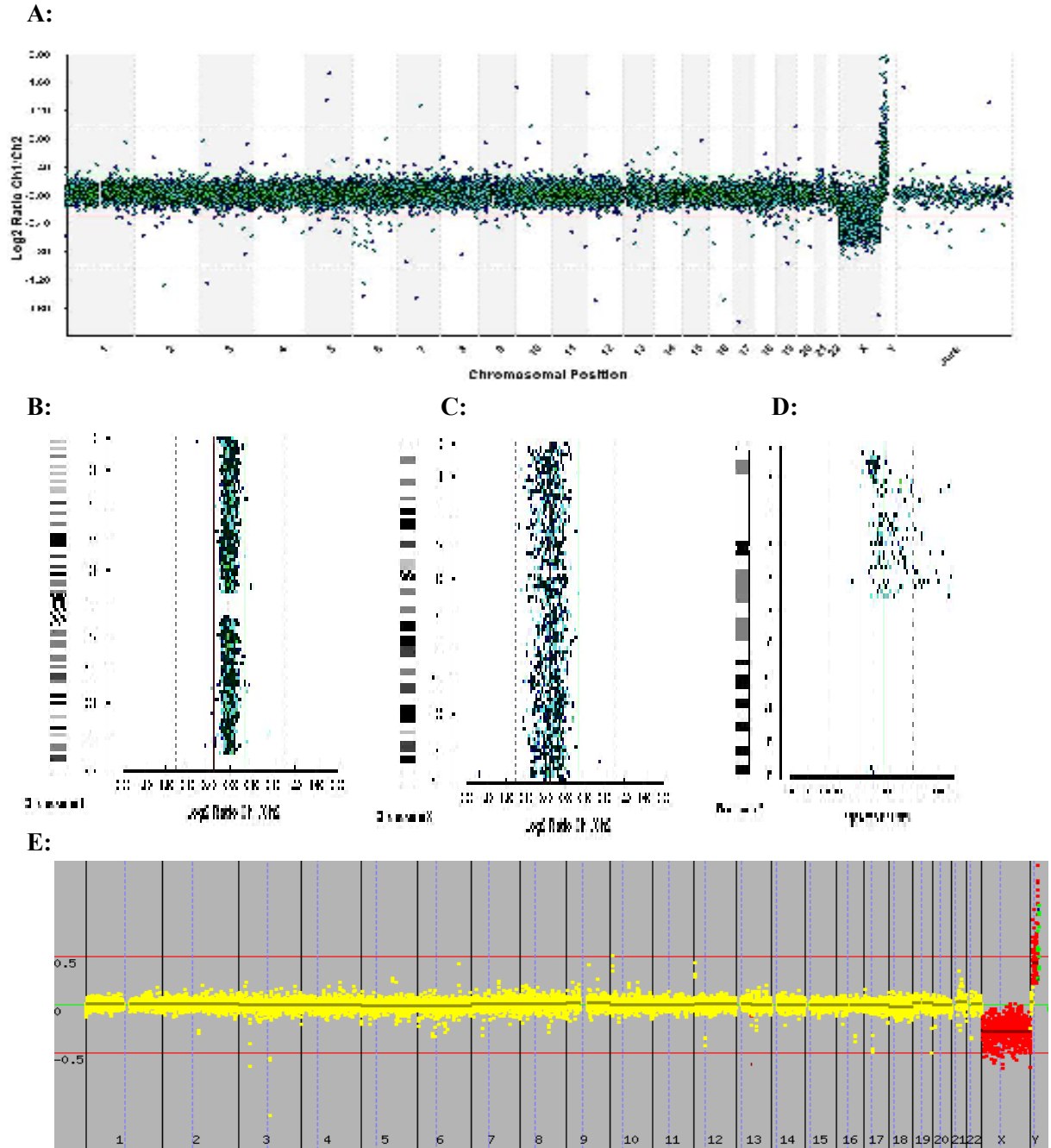


**Figure 4.5: Array Genomics experiment 2 ideograms.** Figure A shows the result from Array Genomics a-CGH slide. Only 56.65% of the clones were included in the analysis. No clones were included for the Y chromosome and only two clones for the chromosome 21. A deletion for chromosome X and amplification for chromosome 13 (Figure B) was scored correctly.



**Figure 4.6: Fibroblast FISH analysis.** FISH analysis for fibroblasts with trisomy 13 (A) and 21 (B). Two hundred cells from each cell line were counted to confirm trisomy uniformity and to exclude mosaicism. Uniformity for T13 line: 97% (194/200), uniformity for T21: 91% (182/200). Probes for chromosome 13: Spectrum Green, probes for chromosome 21: Spectrum Red.

The TRL Mermaid 32K BAC array was tested on good quality gDNA using normal male and female as test and reference samples respectively. The result showed a clear sex mismatch (SD of autosomes: 0.116, SD of X chromosome: 0.218, SD of Y chromosome: 0.502) (figure 4.7). The experiments with these arrays were not performed as dye swap because the cost would increase considerably. Analysis with different software developed from the TRL group was also performed (figure 4.7E).

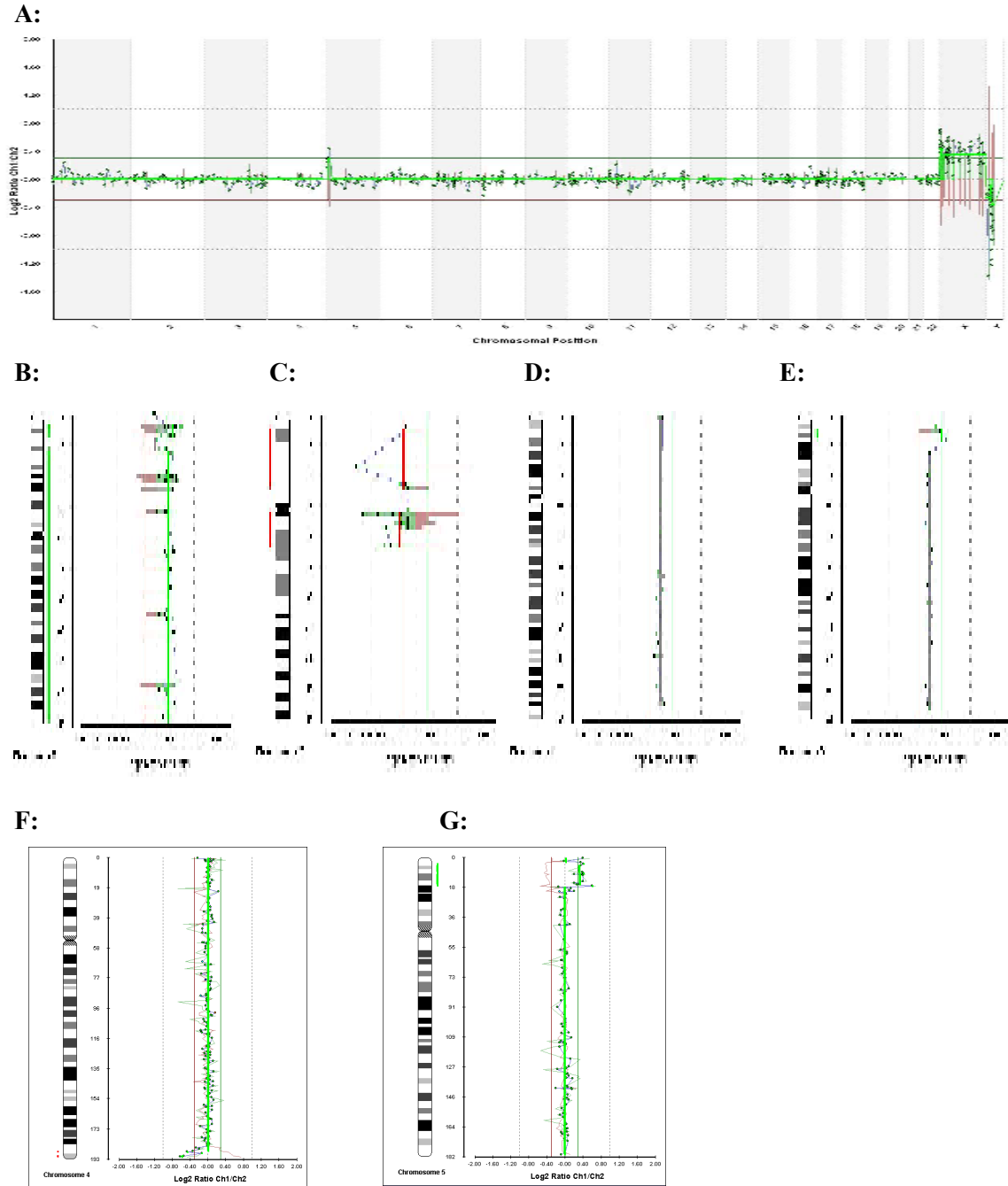


**Figure 4.7: TRL Mermaid arrays experiment B5.** These figures show the result of the Mermaid arrays after hybridisation of good quality gDNA. A clear sex mismatch was detected. A: Graph of all chromosomes after analysis with BlueFuse software, B, C and D: Ideograms of chromosomes 1, X and Y respectively, E: Analysis with a different software.

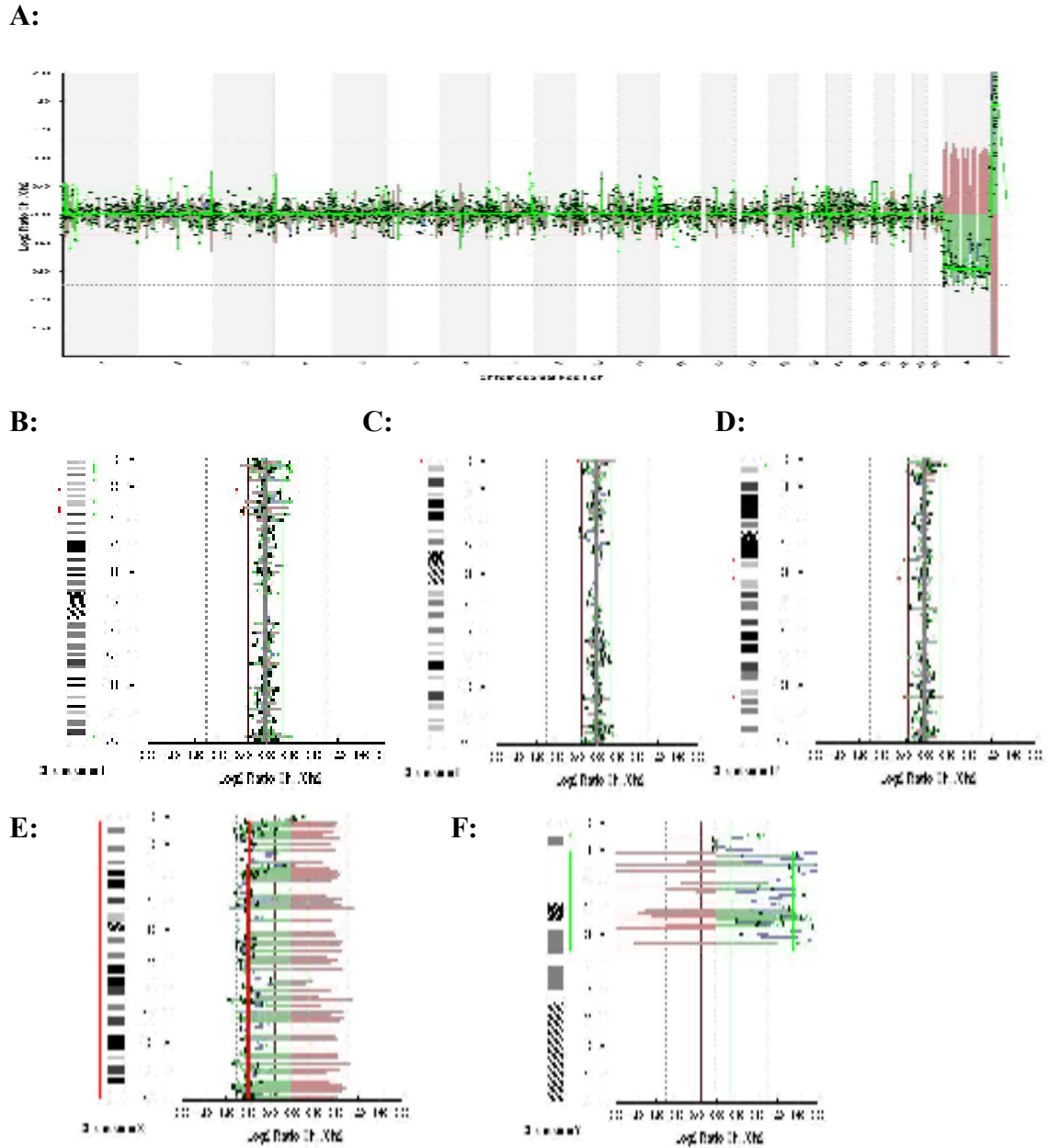


The BlueFuse Cytochip prenatal Beta slide was tested on gDNA. The 1MB Cytochip slides were routinely used in the north Thames Regional Cytogenetic laboratory. The sample used as test DNA was shown to have chromosomal imbalance (46, XY der(4) + (4;5)(q35.1;p14.3)) with the use of the 1MB a-CGH slides and so a replicate experiment was performed with the Cytochip prenatal slide in order to compare the slides (figure 4.8). A very clear sex mismatch was obtained and also the imbalance on chromosome 5 was spotted (SD of autosomes: 0.058, SD of X chromosome: 0.137, SD of Y chromosome: 0.314, SD of chromosome 5: 0.124). However, the deletion on chromosome 4 was not detected with the prenatal slide, which was identified with the 1MB slide. This experiment showed a great sensitivity of Cytochip slides over the other a-CGH slides but also the limitations of using prenatal slides over 1MB slides.

The efficiency of MDA was assessed with the use of the Cytochip 1MB slide (experiment B7). This was performed in order to see if MDA products from initial good quality DNA could be used for a-CGH. The initial concentration of DNA was 20ng for test and reference DNA and both of them were amplified using MDA P3 protocol (section 2.4, table 2.2), MDA for gDNA, two hours incubation). The analysis of this result is shown in figure 4.9. A clear sex mismatch was detected but also high background noise was produced by MDA (SD of autosomes: 0.133, SD of X chromosome: 0.225, SD of Y chromosome: 0.926). This resulted in a number of individual clones to exceed the threshold log<sub>2</sub> intensity ratio of ch1/ch2 which would normally be considered as chromosomal aberrations. Most of these clones were located close to the end of chromosomes. Some regions of the genome, like the 1p36.6 region, were repeatedly shown as imbalanced when Cytochip slides were used with WGA products (other results demonstrate this on graphs from experiments C7 and C8 and from chapter 5). The more profound outcome regarding this experiment was that the resolution of a-CGH dropped with MDA as amplification or deletion of individual clones would be considered as an artefact and the theoretical 1MB resolution could not be achieved.



**Figure 4.8: Cytochip prenatal ideograms of a-CGH experiment B6.** The graph (A) and the ideograms of chromosomes X (B), Y (C), 4 (D) and 5 (E) show the result of gDNA hybridisation on the Cytochip prenatal slides. Sex mismatch is detected as well as duplication in the proximal end of chromosome 5p but no deletion on chromosome 4q is identified. The duplication on chromosome 5p but also the deletion on chromosome 4q was detected when using the Cytochip IMB a-CGH slides (ideograms F and G).



**Figure 4.9: Cytochip 1MB ideograms from experiment B7 (MDA product from gDNA).** The figures show the result after analysis of MDA product from good quality DNA on 1MB a-CGH slide with BlueFuse analysis software. A: Graph of the whole genome. B, C, D, E and F: Detailed ideograms of chromosomes 1, 9, 12, X and Y. A clear sex mismatch is seen but many individual clones on all chromosomes would erroneously show amplification or deletion. These regions are usually located in the chromosome arms.

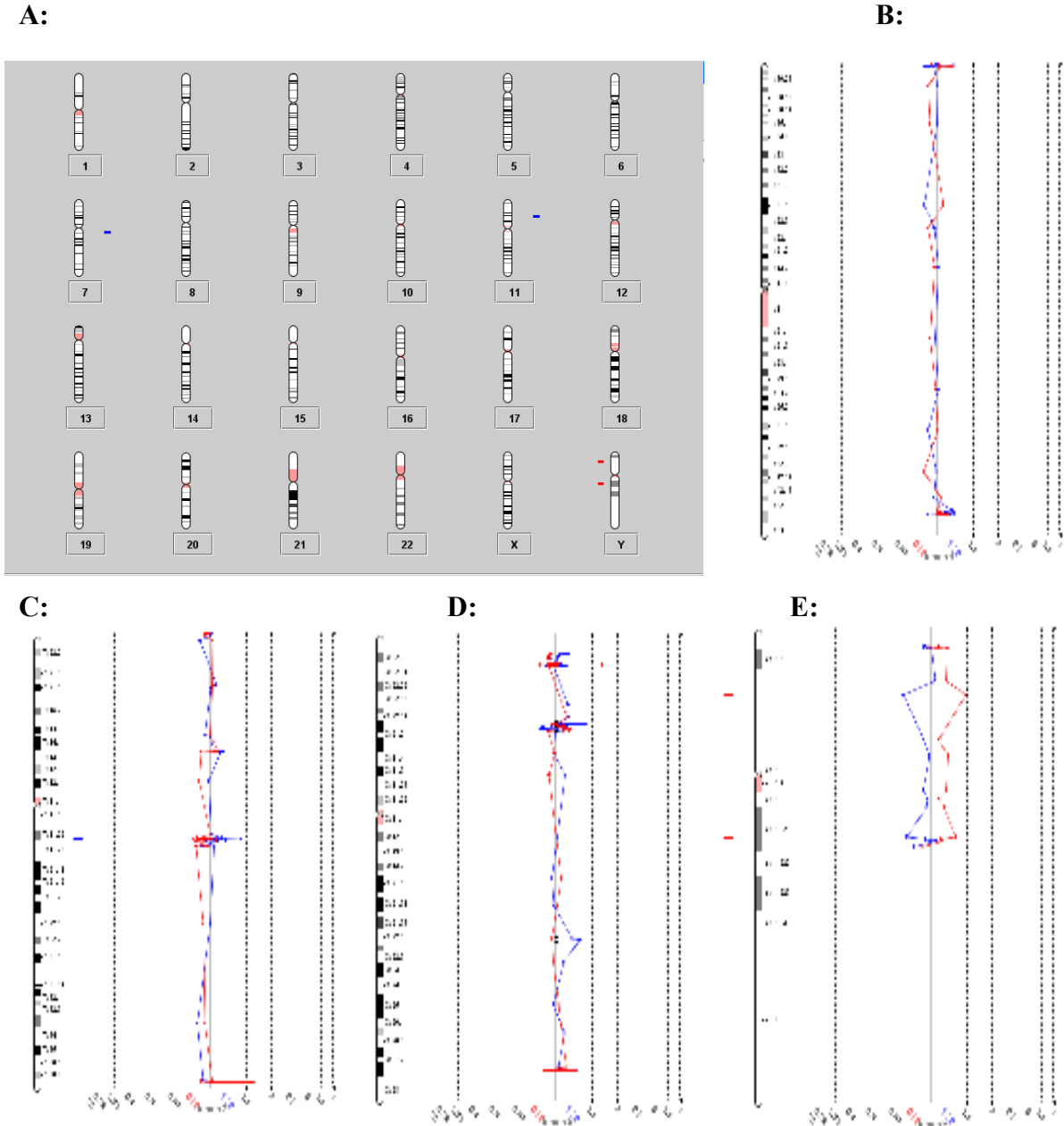
#### 4.6.4 A-CGH results with WGA products from single lymphocytes and fibroblasts

Following the experiments with gDNA, nine experiments were conducted with WGA products from single lymphocytes and fibroblasts. MDA protocol P2 and GenomePlex protocols P6 and P7 were used to amplify the DNA of the single cells. A-CGH slides from Spectral Genomics, Array Genomics, TRL and BlueGnome were used. Table 4.6 lists all the experiments conducted with single cell lymphocytes and fibroblasts.

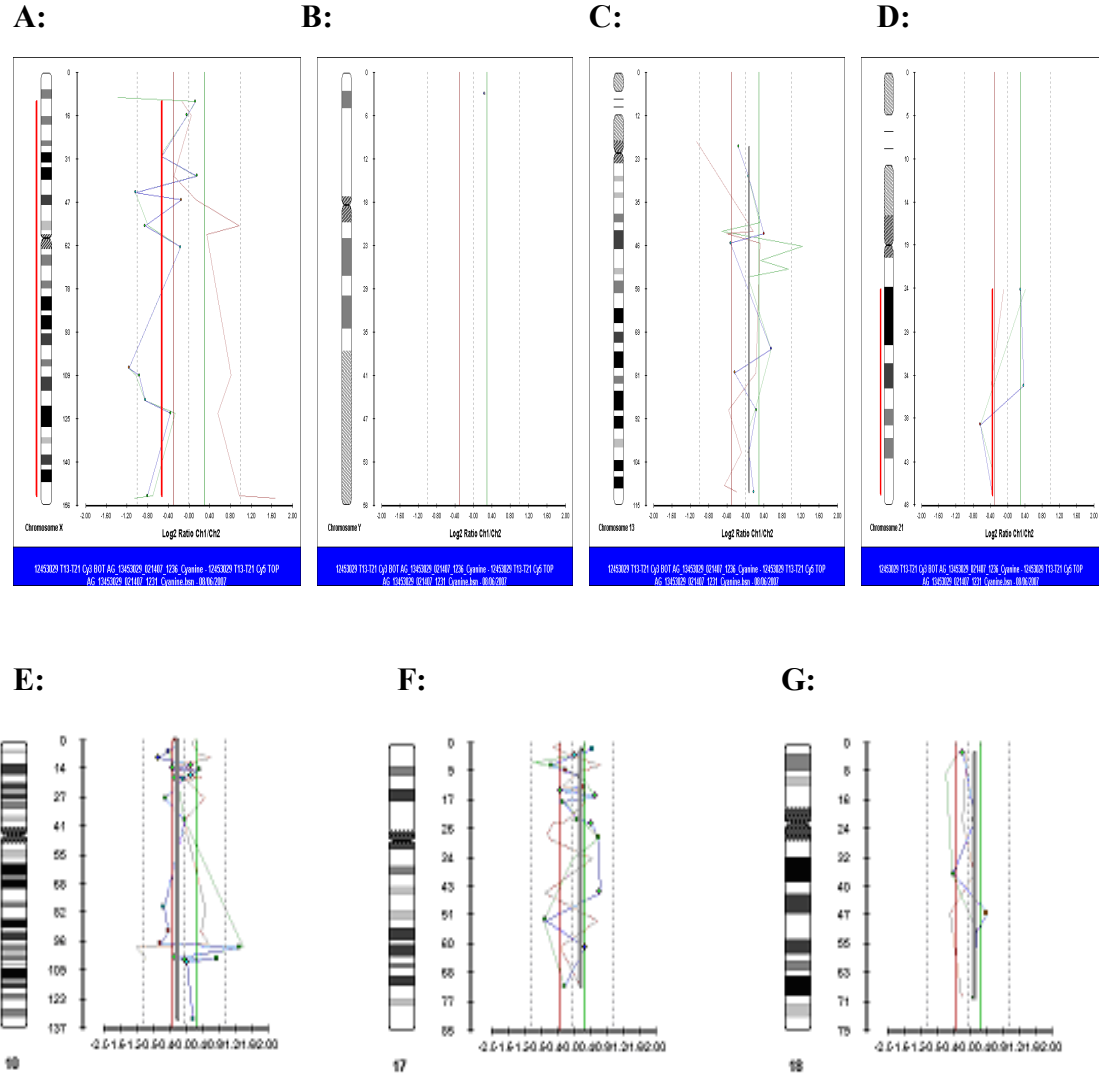
Exper no.	Array slide	Test DNA (single cell WGA)	Reference DNA	Spots analysed	Result
C1	Spectral Genomics 2.0 <sup>TM</sup>	lymphocyte female 7 – P7	Single lymphocyte male 7 – P7	76.75%	No imbalance
C2	Spectral Genomics 2.0 <sup>TM</sup>	fibroblast, T13 male - P2	Single fibroblast, T21 female – P2	19.38%	inconclusive
C3	Array Genomics prenatal	fibroblast, T13 male - P2	Single fibroblast, T21 female – P2	39.25%	inconclusive
C4	Mermaid 32K (TRL)	lymphocyte male 7 – P7	Single lymphocyte female 7 – P7	63.53%	No imbalance
C5	Mermaid 32K (TRL)	lymphocyte female 5 – P6	Single lymphocyte male 6 – P6	15.30%	Inconclusive
C6	Cytochip 1MB (BlueGnome)	lymphocyte male 3 – P2	Female gDNA, No WGA	66.47%	Sex mismatch
C7	Cytochip prenatal (BlueGnome)	lymphocyte male 3 – P2	Single lymphocyte female 1 – P2	57.79%	Sex mismatch
C8	Cytochip prenatal (BlueGnome)	lymphocyte male 7 – P7	Single lymphocyte female 7 – P7	86.14%	Sex mismatch
C9	Cytochip prenatal (BlueGnome)	lymphocyte male 6 – P6	Single lymphocyte female 5 – P6	88.69%	Sex mismatch

**Table 4.6: Experiments performed with WGA products from single lymphocytes and fibroblasts.** This table summarises all the experiments with WGA products from single lymphocytes and fibroblasts together with their results. T21: Trisomy 21, T13: Trisomy 13.

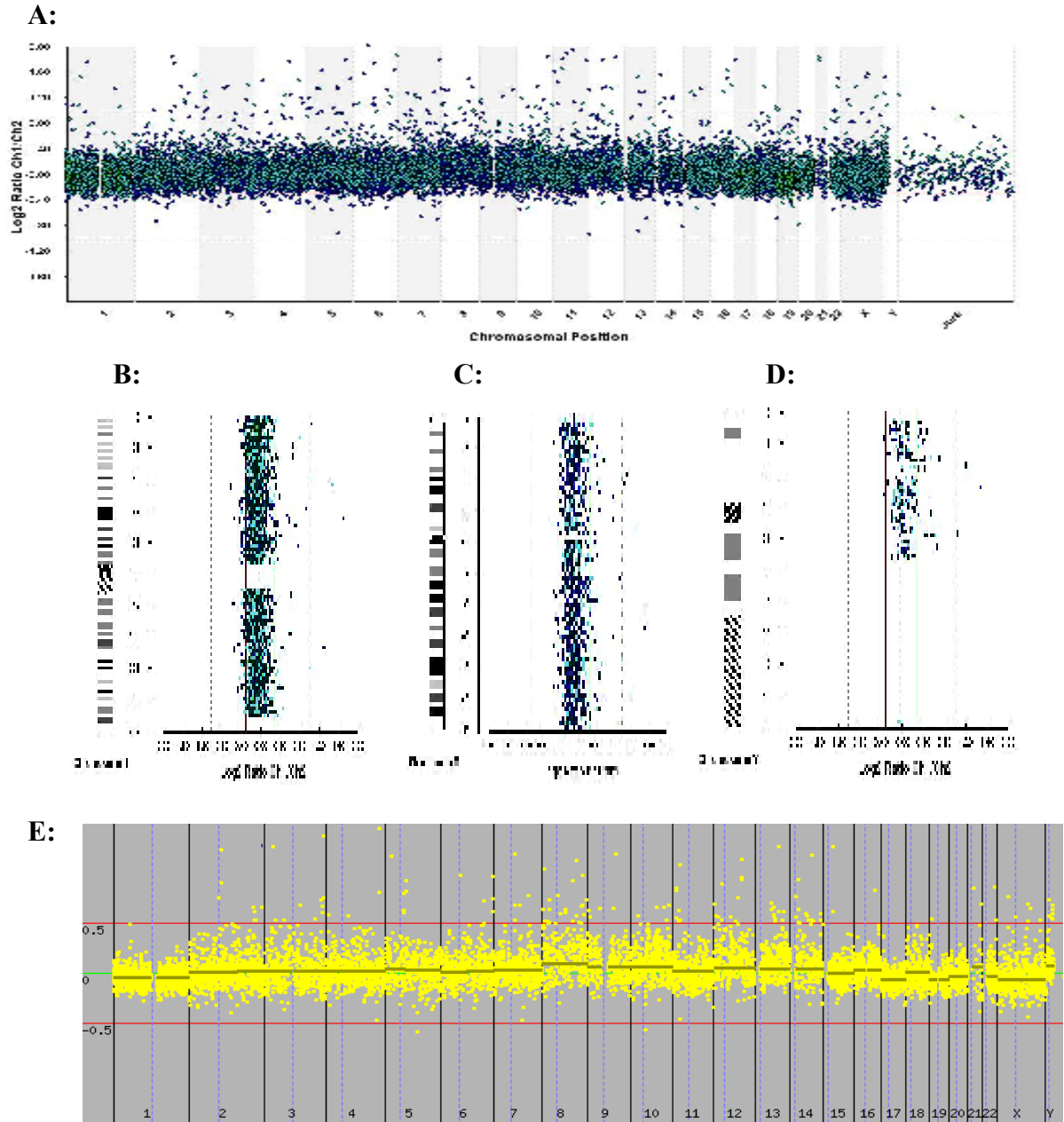
Unfortunately only the BlueGnome Cytochip 1MB and prenatal slides managed to show the expected results. Spectral Genomics, Array Genomics and Mermaid a-CGH slides failed to show any reliable result from three different methods of amplification. Results from these a-CGH slides are shown in figures 4.10, 4.11 and 4.12.



**Figure 4.10: Spectral Genomics constitutional 2.0™ experiment C1.** These figures show the result from MDA product from single lymphocytes with the Spectral Genomics a-CGH slides. Graph A shows that only a partial deletion (two clones only) of chromosome Y is observed. (B): Chromosome 1, (C): Chromosome 7, (D): Chromosome X and (E): Chromosome Y ideograms are displayed.



**Figure 4.11: Array Genomics prenatal slide result of experiment C3.** The ideograms of seven chromosomes from the analysis of MDA products from single fibroblasts on Array Genomics slide are displayed. Although four chromosomal imbalances were expected to be detected, only chromosome X produced a true result. Only one clone was included for the Y chromosome and three from chromosome 21. Generally, the experiment had many artefacts and so the result was inconclusive. Ideograms of chromosomes X (A), Y(B), 13 (C), 21 (D), 10 (E), 17 (F) and 18 (G) are shown.

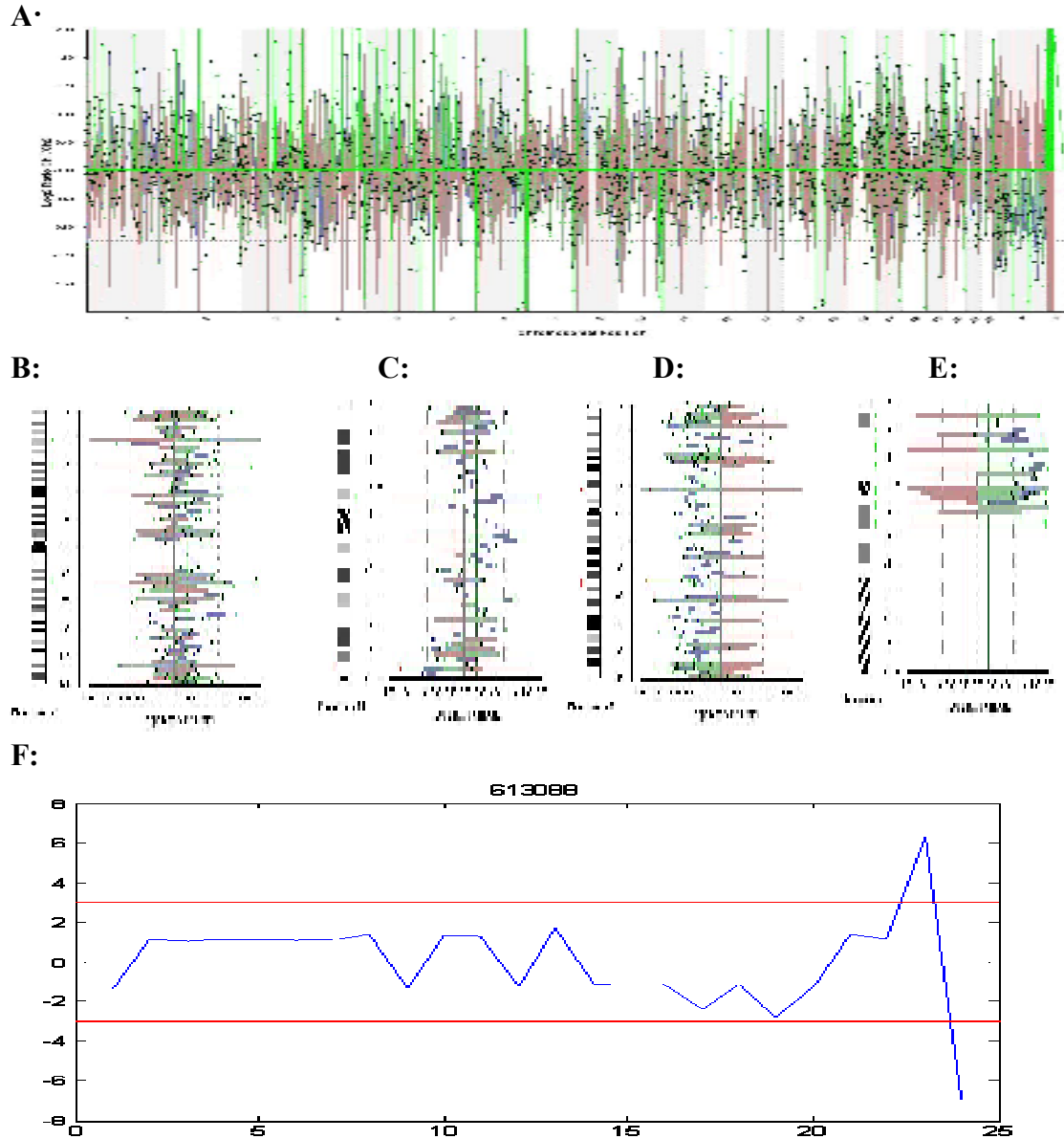


**Figure 4.12: TRL Mermaid array slides of experiment C4.** This figure shows the ideograms from the single lymphocyte cell amplified with GenomePlex (P7 protocol, ALB lysis). No clear difference could be observed for any chromosomes, not even for the Y chromosome where the DNA ratio between test and reference was 1:0. The SD was high for all chromosomes (average SD for autosomes: 0.235). A: Graph of the result. B, C and D: Ideograms of chromosomes 1, X and Y respectively. E: Analysis with other software than BlueFuse.

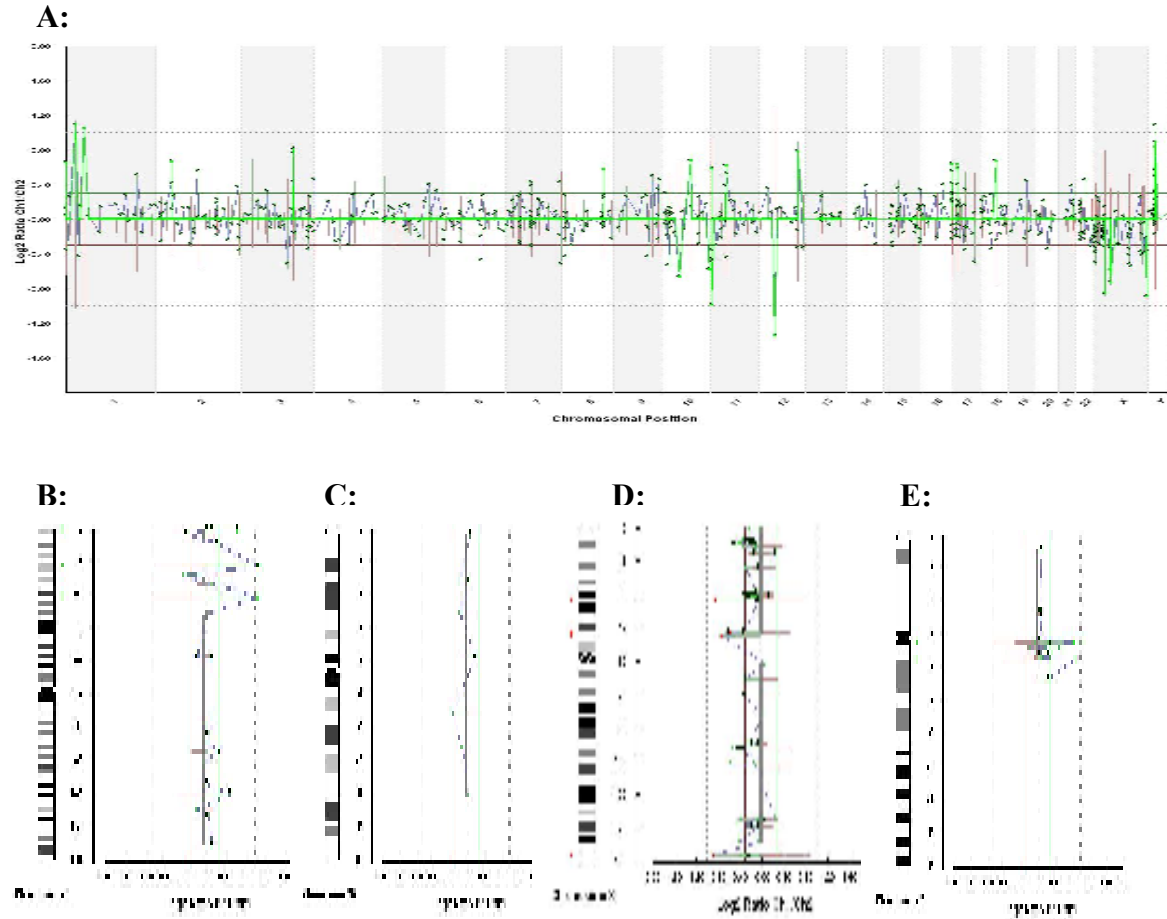
The BlueGnome Cytochip 1MB slide was used to test MDA product from single male lymphocytes against good quality female gDNA. The results are shown in figure 4.13. Although the MDA products produced very high background noise (SD for autosomes: 0.642, SD for X chromosome: 0.577, SD for Y chromosome: 0.914, clones included: 66.47%, median between replicates: 0.06), a clear amplification on chromosome Y and deletion on chromosome X was observed. The imbalance of the sex chromosomes could not be identified with the use of BlueFuse Cytochip analysis as it is not programmed to obtain results with high SD. Analysis with a different algorithm (prenatal algorithm, currently being developed by BlueGnome) was performed in order to avoid the high background noise and detect whole chromosome changes (figure 4.13F). In this graph the X chromosome appeared as amplified and the Y chromosome as deleted due to the reverse order of the top and bottom sub-array by mistake. Due to the background noise it was concluded that only large/whole chromosome changes could be detected. In order to minimise the background noise in the following experiments single cells were used as reference DNA and were amplified in a similar way as test DNA.

The three following experiments were done using the BlueGnome Cytochip prenatal slides instead of the 1MB slides. That was done in order to further decrease the background noise. Even though the resolution would be decreased the clones were expected to be enough to detect large/whole chromosome changes. In each of these experiments the test and the reference DNA were male and female single lymphocytes were amplified with a different WGA method. In the first experiment MDA P2 protocol was used for WGA whereas in the other two experiments GenomePlex P6 and P7 protocols were used. That was done to compare the two WGA techniques and to see if cell lysis would play a crucial role in the result. A sex mismatch was expected to be detected in each experiment (4.14 and 4.15).

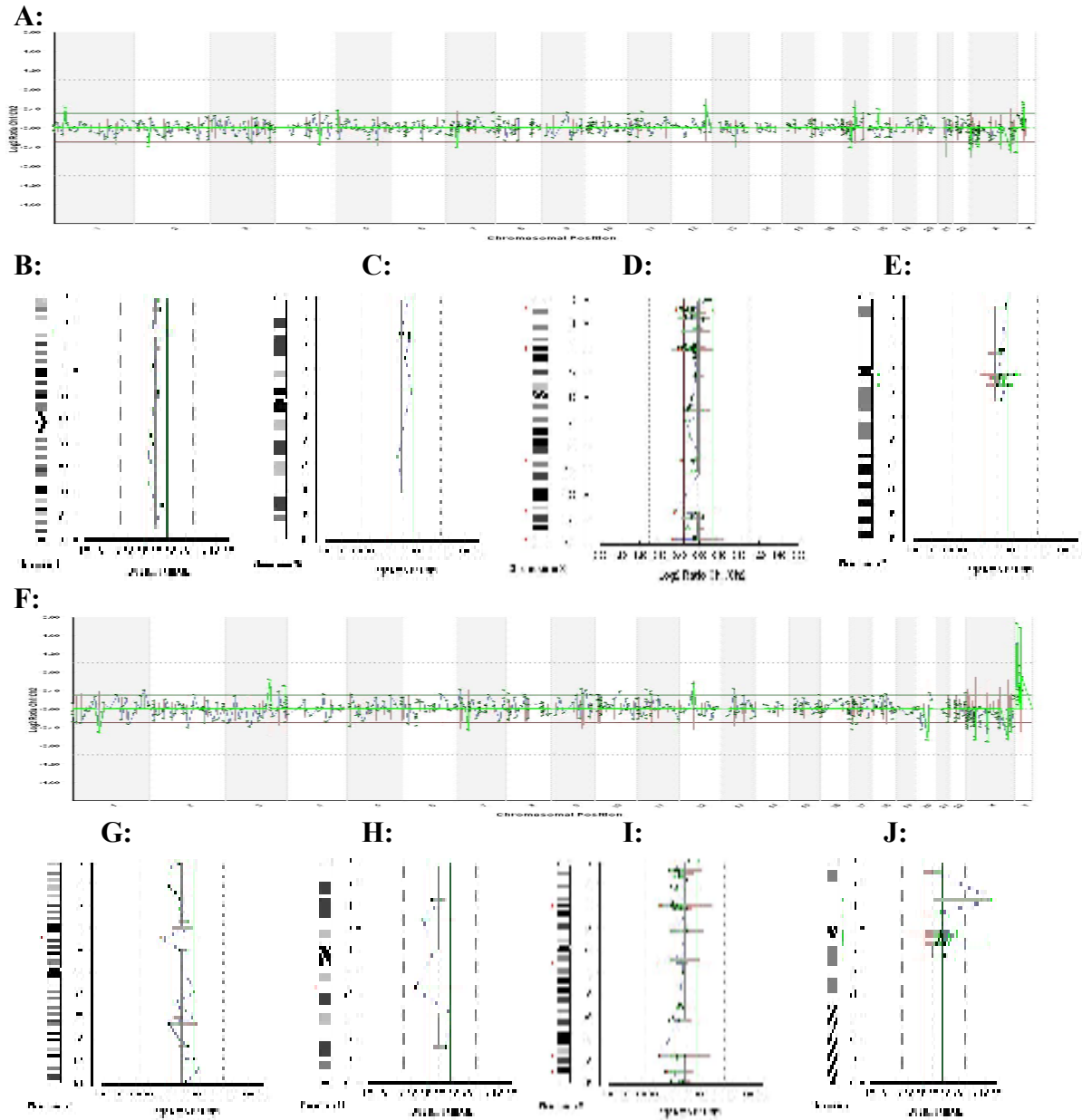




**Figure 4.13: BlueGnome Cytochip 1MB slide of experiment C6.** The figures show the analysis of MDA product from single lymphocyte (male) against high quality gDNA (female) using the Cytochip 1MB slide. Figure A shows the graph of the entire genome. Figures B and C show the ideograms of chromosomes 1 and 20 in which the majority of clones are shown as amplified or deleted. Figure D and E show the ideograms of chromosomes X and Y where the majority of clones were detected as deleted and amplified respectively. Graph F shows the analysis of the result with the prenatal algorithm which shows sex mismatch result in opposite orientation due to the mistake in the analysis software.



**Figure 4.14: BlueGnome Cytochip prenatal slide Ideograms of experiment C7 (MDA P2 protocol).** These figures present the result from MDA products from single lymphocytes (test and reference DNA) on a Cytochip prenatal slide. Graph A shows the result of the entire genome, figures B and C the ideograms of chromosomes 1 and 20. Although the background noise was high sex mismatch could be detected as the vast majority of clones for chromosome X and Y appeared as deleted and amplified respectively (D and E). Many individual clones would exceed the threshold ratio for chromosome imbalance (A), such as region 1p36.



**Figure 4.15: BlueGnome Cytochip prenatal results of experiments C8 and C9 (GenomePlex P6 and P7 protocols).** Figures A- E: Graph of Cytochip prenatal slide of single cell GenomePlex products with P6 protocol (proteinase K lysis) and ideograms of chromosomes 1, 20, X and Y respectively. Figures F-J: Graph of Cytochip prenatal slide of single cell GenomePlex products with P7 protocol (ALB L1 lysis) and ideograms of chromosomes 1, 20, X and Y. Both results have decreased background noise compare to MDA products. GenomePlex with ALB lysis had more noisy results but clearer results for sex mismatch.

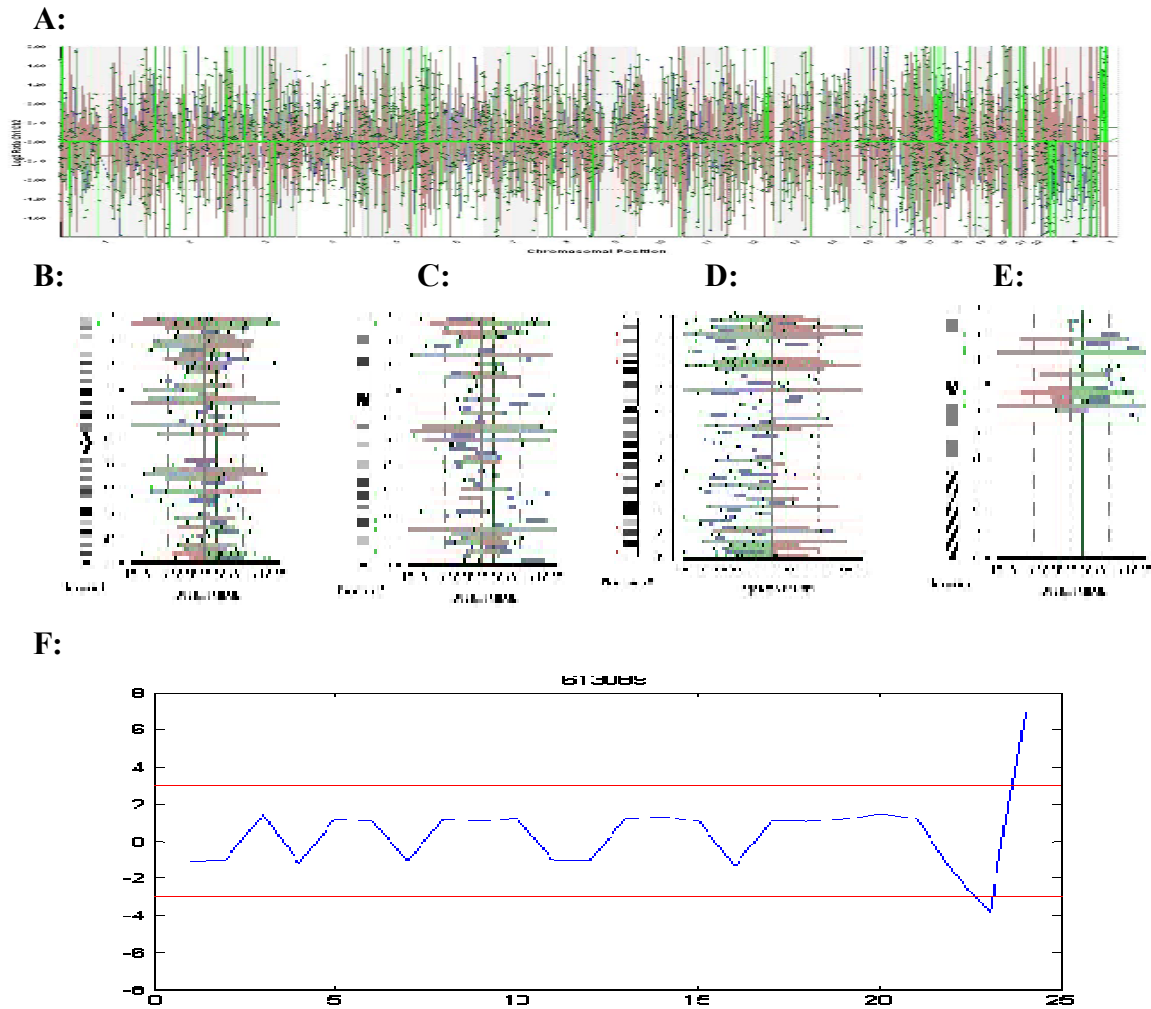
Among the three results the experiment with the MDA products had the highest SD and the highest median between replicates (SD for autosomes: 0.243, SD for X chromosome: 0.205, SD for Y chromosome: 0.293, clones included: 57.79%, median between replicates: 0.13) compared to the GenomePlex products lysed with Proteinase K (SD for autosomes: 0.127, SD for X chromosome: 0.142, SD for Y chromosome: 0.128, clones included: 86.14%, median between replicates: 0.08) and the GenomePlex products lysed with ALB (SD for autosomes: 0.168, SD for X chromosome: 0.179, SD for Y chromosome: 0.382, clones included: 89.22%, median between replicates: 0.07). Although only one result per WGA protocol was obtained which was not very secure for conclusions, GenomePlex with ALB lysis was considered to provide the most desired outcome compare to the other methods.

Generally, from the single cell a-CGH results only Cytochip prenatal and 1MB resolution slides managed to show at least sex mismatch difference even though the background noise was very high. Cytochip prenatal slides decreased the background noise but would only be reliable for whole chromosome changes. Analysis with a different algorithm could be the key for more secure outcomes.

#### **4.6.5 A-CGH results with WGA products from single blastomeres**

Following the single cell a-CGH project with lymphocytes, the application of single blastomeres to a-CGH was tested. The WGA products were applied on the BlueGnome Cytochip 1MB slide in order to see if any chromosomal abnormalities could be detected. Although the Cytochip prenatal slides provided lower background noise, the 1MB slides were preferred because there was a higher chance to detect a chromosome abnormality whereas analysis with a different algorithm could help to overcome the noisy background. A total of five blastomeres were obtained from three embryos. The first embryo was from a PGD case for fragile X syndrome, where male embryos were excluded from transfer. A blastomere was biosied from a day 5 embryo of high quality (4AA) and MDA was applied (protocol P2). The rest of the embryo was disaggregated and the blastomeres were fixed on a poly-lysine slide by Dr Anna Mantzouratou and

FISH was applied for chromosomes 13, 21, 15, 18, X and Y and no chromosomal abnormality was detected. The result of the a-CGH experiment is shown on figure 4.16 (experiment D1). The result suffered from very high noise (SD for autosomes: 0.840, SD for X chromosome: 0.884, SD for Y chromosome: 1.034, clones included: 71.30%, median between replicates: 0.06) but sex mismatch could be detected when analysed with the prenatal algorithm.



**Figure 4.16: BlueGnome Cytochip 1MB slide result of experiment D1 (Embryo 1, blastomere 1, MDA).** The results were very noisy (Graph A). High SD was obtained for all chromosomes, like chromosomes 1 (B) and 17 (C) although a clear deletion and amplification of chromosomes X and Y respectively was also obtained with the original analysis (D and E). Analysis with the prenatal algorithm showed a clear chromosomal change on chromosomes X and Y (F).

The second embryo was obtained from a PGS case where FISH analysis on a single blastomere during the clinical case resulted in one signal for chromosome 21 and two for chromosomes 13, 18, 15, 16 and 22. The quality of the embryo was also reported to be low by the embryologists (2CC). After exclusion from transfer, two blastomeres were biopsied, one of them was subjected to MDA (protocol 2) and one was subjected to GenomePlex (protocol 7, ALB lysis). The rest of the embryo was spread on a poly L-lysine coated slide for FISH. The MDA product was subjected to PCR for the DM triplex and ensured that the reaction amplified the DNA successfully (table 4.7). Other markers on various chromosomes were used as well for confirmation of the a-CGH results (table 4.7). Unfortunately, robust GenomePlex amplification for single cells could not be confirmed with PCR and the success of the reaction only depended on the DNA concentration of the product (43ng/μl, table 4.2). As reference DNA, a single female lymphocyte was used which was amplified in the same way for each experiment.

Markers/Samples	Embryo 2, Blastomere 1	Embryo 3, Blastomere 1
<b>Allele sizes</b>		
<b>Chromosome 5</b>		
D5S1965	180/ 206	205
D5S2065	214	214
D5S656	-	-
D5S346	251	251
<b>Chromosome 7</b>		
p.Phe508del	94	94
IVS8CA	185	182
<b>Chromosome 19</b>		
APOC2	150	135 /150 /154
DM1	128/ 146	128 /146 /181
D19S112	117	117/ 130
<b>Sex chromosomes</b>		
Amelogenin	104	104/ 110

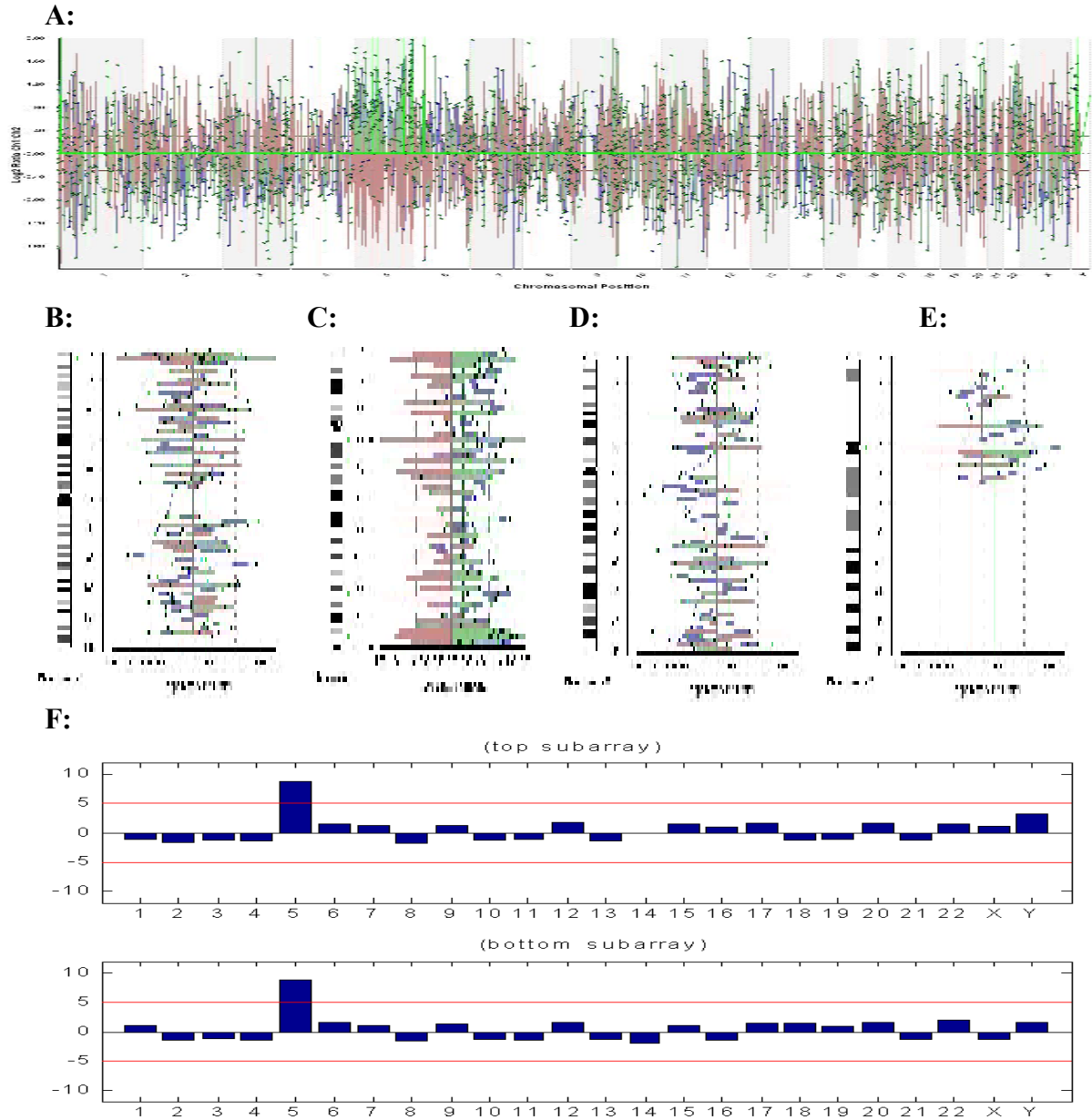
**Table 4.7: PCR analysis with 10 markers on five chromosomes for blastomeres.** This table summarises the results obtained from analysis at 10 loci on the MDA products from blastomeres prior to a-CGH. Three alleles were found in two markers of chromosome 19 for embryo 3, blastomere 1 and a-CGH confirmed a trisomy for this chromosome. Only one marker (D5S656) did not show any results for both MDA products even though it worked on the positive control.

For the D2 experiment the background noise produced by the MDA product was very high (SD for autosomes: 0.693, SD for X chromosome: 0.676, SD for Y chromosome: 0.728, clones included: 49.35%, median between replicates: 0.15) and analysis with the prenatal algorithm was also performed which revealed an amplification for chromosome 5 (47, XX +5) (figure 4.17). This time the prenatal algorithm was applied without the use of dye swap and each sub-array was considered as a different experiment in order to confirm reproducibility of the results. For the GenomePlex product the hybridisation was very poor (31.31%) which also resulted in high background noise (SD for autosomes: 0.333, SD for X chromosome: 0.293, SD for Y chromosome: 0.374, median between replicates: 0.22). Although analysis with the prenatal algorithm was performed no chromosome imbalance was detected (figure 4.18).

Follow up FISH analysis for the same six chromosomes characterised the embryo as chaotic whereas a third round of FISH for chromosomes 1, 5, and 19 failed due to poor hybridisation. PCR analysis on the MDA product was also performed with a total of seven markers on chromosomes 5, 7, X and Y (table 4.6). Interestingly none of the four markers for chromosome 5 had three alleles. Thus the PCR results could not confirm or reject the presence of trisomy 5. One allele was detected for the amelogenin gene which confirmed the result of a-CGH that the embryo was female.

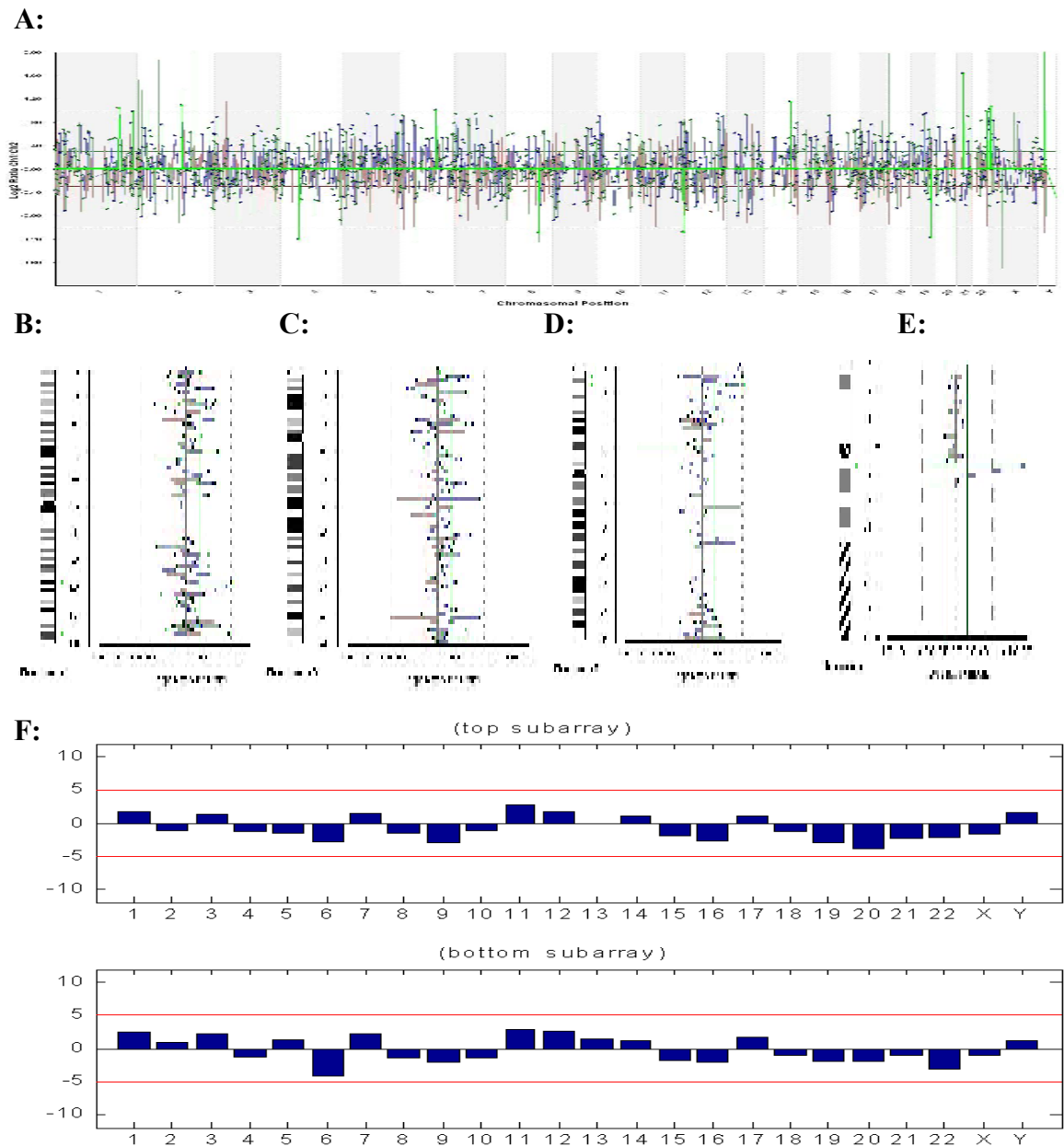
The third embryo was from the same cohort as the second embryo. FISH analysis from the PGS case on a single blastomere resulted in one signal for chromosomes 13, 16 and 21 two signals for chromosomes 15 and 22 and three signals for chromosome 18. Again, the quality of the embryo was low (2CB). After exclusion from transfer, two blastomeres were biopsied, one of them was subjected to MDA (protocol 2) and one was subjected to GenomePlex (protocol 7, ALB lysis). Similarly to the previous experiment, the rest of the embryo was spread on a poly L-lysine coated slide for FISH and the MDA product was subjected to PCR for the DM triplex. FISH analysis on the same six markers characterised the embryo as aneuploid/chaotic mosaic with monosomy 21 due to meiotic error (90% monosomy for chromosome 21). PCR for DM locus on MDA product created suspicions of trisomy 19 as two of the three markers presented three alleles (table 4.6).





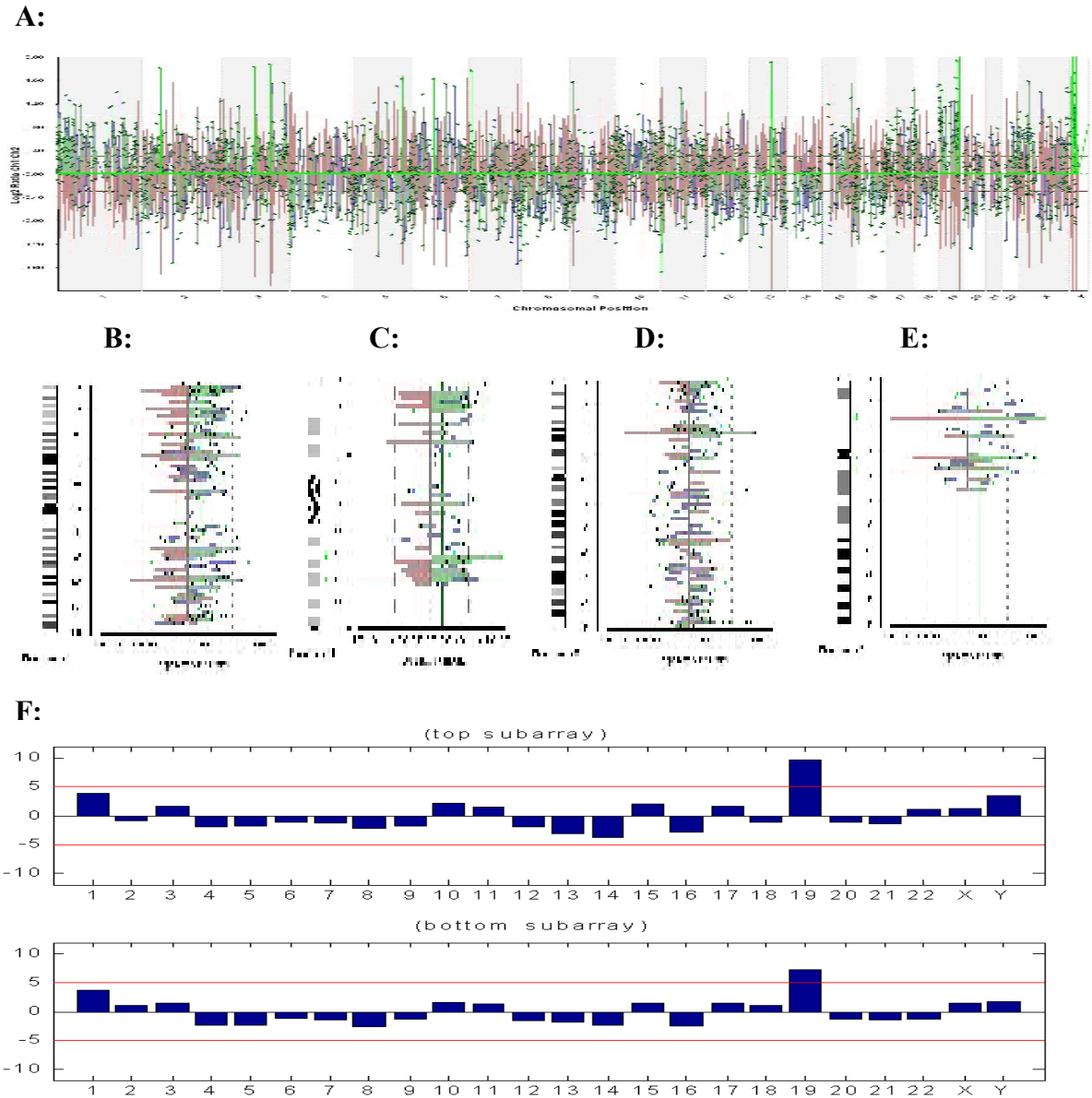
**Figure 4.17: BlueGnome Cytochip 1MB slide result of experiment D2 (Embryo 2, blastomere 1, MDA).** The figures show the a-CGH analysis from blastomere 2 after MDA. Graph (A) shows the result of the entire genome. Figures B, C, D and E show the ideograms of chromosomes 1, 5, X and Y chromosomes respectively. The majority of clones of chromosome 5 were seen as amplified. Analysis with the prenatal algorithm was done separately on each sub-array which demonstrated the reproducibility of the result (F). The application of the prenatal algorithm showed a clear chromosomal change on chromosomes 5 (F) on both sub-arrays.





**Figure 4.18: BlueGnome Cytochip 1MB slide result of experiment D3 (Embryo 2, blastomere 2, GenomePlex).** The figures show the analysis from blastomere 3 after GenomePlex. Due to very poor hybridisation only 31.31% of the clones were used for the analysis which makes the result very unreliable. No chromosomal imbalances were obtained for any chromosomes. A: Graph of the entire genome. B, C, D and E: Ideograms of chromosomes 1, 5, X and Y. F: Analysis with the prenatal algorithm.

The results from the a-CGH experiment are shown on figure 4.19 (experiment D4). Unfortunately, the GenomePlex amplification failed (concentration of product = 6ng/μl) and so results only from the MDA product were obtained. The analysis with the prenatal algorithm was necessary due to the high background noise (SD for autosomes: 0.519, SD for X chromosome: 0.511, SD for Y chromosome: 0.861, clones included: 49.73%, median between replicates: 0.14). A trisomy on chromosome 19 was detected in both sub arrays (47, XX +19). Trisomy 19 was also confirmed from the PCR analysis on the DM locus but analysis of the sex chromosomes showed that the blastomere was male (table 4.7). Additionally, the majority of clones on chromosome 1 were detected as amplified which implied that there was an imbalance for this chromosome (figure 4.19D). However, the analysis with the prenatal algorithm did not detect any imbalance for chromosome 1. A third round of FISH for chromosomes 1, 5 and 19 on the rest of the embryo failed due to poor hybridisation which could have given some information for chromosome 1 in the rest of the blastomeres. Unfortunately, there were not any STR markers for chromosome 1 that could be used for PCR analysis on the MDA product. Table 4.8 summarises all the results from a-CGH, FISH results from clinical PGS and follow up results and PCR on markers on chromosomes 5, 7, 19, X and Y.



**Figure 4.19: BlueGnome Cytochip 1MB slide result of experiment D4 (Embryo 3, blastomere 4, MDA).** Graph A show the analysis from the first blastomere after MDA of embryo 3. Detail analysis of chromosomes 1, 19, X and Y are displayed on figures B, C, D and E respectively. Analysis with the prenatal algorithm detected amplification of chromosome 19 for both sub-arrays. However, most clones of chromosome 1 were detected as amplified which increased the probability of a trisomy 1.

Exper no.	Test DNA	Ref. DNA (single cell)	WGA	a-CGH result	Clinical PGS	FISH on rest of embryo	PCR analysis
D1	Embryo 1 blast. 1	Lymphocyte female	MDA	46, XX	Not performed	No abnormality detected	Not performed
D2	Embryo 2 blast. 1	Lymphocyte female	MDA	47,XX+5	Monosomy 21	Chaotic	Female, No trisomy 5 detected
D3	Embryo 2 blast. 2	Lymphocyte female	GenomePlex	poor hybridisation			
D4	Embryo 3 blast. 1	Lymphocyte female	MDA	47,XX+19	Chaotic	Chaotic/ monosomy 21	Male, Trisomy 19

**Table 4.8: Summary of results obtained from WGA products from single blastomeres.** This table compares the results obtained from a-CGH with the results from FISH and PCR. Direct comparison cannot be performed with FISH because different chromosomal abnormalities can be obtained from blastomeres of the same embryo. However, a trisomy 19 detected by a-CGH was confirmed by PCR.

#### 4.7 Discussion

A-CGH is a very advanced technique with great potential for both research and diagnostic purposes. However, application of single cells on a-CGH has technical limitations. Very few studies on single cell a-CGH have been published due to these problems. The most profound problem is the background noise which makes analysis more complicated. In this project, two different WGA techniques were used to amplify the entire genome from a single cell and applied to BAC a-CGH slides. Wherever it was possible, opposite sex of test and reference samples were chosen as a positive control. Most of the experiments were performed using the BlueGnome Cytochip 1MB or prenatal slides as these were the ones that showed the best results on gDNA and single cell experiments. Two lysis methods were performed prior to GenomePlex; one was proteinase K lysis as obtained from the manufacturers' instructions and one was L1 lysis which was found to perform better than MDA (chapter 3).

In the first application of single cell a-CGH, a different platform and amplification method was used (Hu et al., 2004). The limitations of this project were that although

expected trisomies were detected from fibroblast cell lines, the result depended on comparing the intensity ratios from the aneuploid and the euploid chromosomes. An additional problem was the inability to perform correct analysis for the Y chromosome, even though correct results were obtained for the X chromosome. Hellani et al. (2004) reported single cell a-CGH using MDA and the Spectral Genomics 1MB a-CGH. In contrast to the results obtained from this project with the Spectral Genomics array slides, a trisomy 21 was detected even though only half of the clones of chromosome 21 exceeded the Ch1/Ch2 ratio threshold. In the following years four more groups published their results (Le Caignec et al., 2006; Fiegler et al., 2007; Iwamoto et al., 2007; Fuhrmann et al., 2008). In these projects different WGA techniques, slide resolution and array platforms were used but all the results were promising. The resolution analysis from these papers ranged from 34MB up to 2-3MB. Very recently the first clinical application of PGS using a-CGH was published concentrating only in whole chromosome changes (Hellani et al., 2008). All these papers are discussed and compared with the results from this project.

#### **4.7.1 A-CGH platforms**

Three major different types of a-CGH platforms have been constructed from different companies. The main difference is the DNA probes that bind on the glass slide which can be DNA from chromosome-specific DNA libraries, BAC probes or oligonucleotides (25-85) synthesized in situ. Currently all these platforms have been used for single cell a-CGH but most of the published work is based on BAC a-CGH slides. Each platform has advantages and disadvantages while the most promising results are from oligonucleotide and SNP arrays with the crucial advantage being the reduced background noise. In the first published paper in 2004 (Hu et al., 2004) chromosome-specific DNA libraries were used and this was followed by four papers using BAC array platforms (1MB and 100K tiling-path) (Hellani et al., 2004; Le Caignec et al., 2006; Fiegler et al., 2007; Fuhrmann et al., 2008) and two with oligonucleotide array platforms (Iwamoto et al., 2007; Hellani et al., 2008).

A different platform is not the only factor affecting the outcome of single cell array experiments. Even when using slides from the same platform from a different company the results can vary. An example is the results obtained from this project where the Spectral Genomics constitutional slides, the Array Genomics prenatal slides and the BlueGnome Cytochip prenatal slides had a very similar manufacturing principle and resolution but different outcome from the same WGA products. Differences could exist from the way each company amplifies the DNA from the BAC probes. Slides from the Sanger Institute and BlueGnome used DOP-PCR for their BAC probes whereas TRL used MDA. No information was given by Spectral Genomics and Array Genomics about the amplification method of the target DNA. Spotting techniques, probe diameter and distance between spots could also play a role in the result. A big difference was noted in the probes spotted from the TRL tiling-path arrays and the other companies as in the Mermaid 32K arrays the spots were much smaller and closer to each other. In addition the results from the Cytochip 1MB slides had higher SD compared to the Cytochip prenatal slides where the spots were larger and the distance between them greater. The fact that results from the same arrays but from a different batch could lead to different results makes clear that small differences during array fabrication could lead to diverse results (Wells et al., 2008).

Fuhrmann et al. (2008) showed how a modified BAC a-CGH platform could provide high resolution analysis with single cells. The idea was based on the fact that BAC clones and WGA products from single cells would be contaminated by micro-quantities of bacterial DNA and this resulted in unspecific hybridisation on the array slide. Although the normal BAC array slide could not even detect whole chromosome changes, the modified platform which consisted of highly purified BAC clones was able to identify chromosomal gain or loss as small as 4.4MB. Additionally, the exclusion of BAC clones with high GC content (>45%) was found to further improve single cell a-CGH results. This paper would be of great value for further single cell a-CGH research and high resolution analysis. Unfortunately, in this project it was not possible to manipulate the a-CGH slides and so the research was focused only on comparing slides from different companies in order to obtain the best result for single cell a-CGH.

#### **4.7.2 WGA method for test and reference DNA**

So far most WGA techniques have been applied to single cell DNA amplification prior to a-CGH including DOP-PCR, MDA, GenomePlex and LA adaptor PCR. The WGA techniques could mainly be separated in to MDA and PCR-based. The main advantage of the PCR based WGA is that they produce less non-specific DNA and minimise the background noise. The results from the Cytochip prenatal and 1MB slides showed that SD of log2 ratio was higher for the MDA products (0.42-1.3) compared to GenomePlex products (0.38 – 0.4). Similar findings were obtained from the literature where Le Caignec et al. (2006) used MDA and reported an SD of 0.84 – 1.13, whereas Fiegler et al. (2007) used GenomePlex and Fuhrmann et al. (2008) used LA adaptor PCR and obtained SD of 0.14- 0.24 and 0.23 -0.36 respectively from their experiments. Another advantage of the PCR based techniques is that they do not under- or over- amplify regions close to the chromosome ends. The graphs in figure 4.14 show a gain or loss in the chromosome ends in the MDA products whereas this was not observed in the experiments with GenomePlex amplified DNA (figure 4.15). This artefact was more obvious in the 1MB slide compare to the prenatal slide probably due to the higher density of probes in the telomeric regions.

The big advantage of MDA is the production of large DNA molecules that can be used for molecular analysis when the MDA technique has correctly amplified the DNA and so PCR could confirm a result obtained from a-CGH. In this project a trisomy 19 was confirmed with the use of PCR on the DM locus and also a blastomere shown to be female from a-CGH was shown to be male with the use of PCR markers on the amelogenin gene. A different approach could not be used in case of blastomeres in order to confirm the result and so if another WGA method was used instead confirmation of the results would not be feasible. In cases of single cell a-CGH from cell lines or tumour cells FISH analysis on other cells could confirm the array result. Unfortunately, most of the high molecular weight products and the hyper-branched structures produced by MDA are probably discarded during DNA purification as the DNA could be reduced up to 1/3

of the initial volume after DNA clean up. As this could influence the result of an experiment, a single cell a-CGH research project is being carried out currently in UCL Centre for PGD that excludes the DNA clean-up step (Thalia Mamas, personal communication). Mild DNA digestion could also provide a solution to this problem. A modified version of MDA, named restriction and circularization-aided rolling circle amplification (RCA-RCA), could be used as an advanced technique to overcome the difficulty of amplification at the chromosome ends but it would also hamper the advantage of MDA for molecular analysis.

GenomePlex was used because the company provided an optimised protocol for single cells, and also good genome coverage and low amplification bias was obtained from other published work (Arneson et al., 2008). A single cell a-CGH paper was also published with the use of GenomePlex (Fiegler et al., 2007). As cell lysis was shown to play a crucial role in the result of the molecular project the L1 lysis was used in a modified protocol for GenomePlex. Unfortunately, the two lysis methods could only be compared from two experiments (Figure 4.13) which was not enough to make secure outcomes. Nevertheless, from the results obtained alkaline lysis was shown to increase the background noise but sex mismatch was also clearer. Generally, the main drawback of GenomePlex regardless of the lysis method was that only 70% of the cells managed to amplify (table 4.2). Unfortunately, none of the GenomePlex products from single blastomeres showed an array result although better results than the MDA products were expected. More experiments would be needed in order to make a secure result about the reaction's amplification efficiency.

Another factor related to the amplification method of the DNA has to do with the WGA that was used to enrich the DNA of the BAC probes prior to fabrication on the slide. Generally, the same amplification method for the DNA probes and the test and reference DNA should be avoided as it would increase the background noise (Nigel Carter, personal communication). In the results presented in this chapter one experiment was conducted which used the same WGA as the one used to enrich the target DNA (experiment 4) but inconclusive results were obtained due to high SD. For the TRL slides



MDA was used to enrich the target DNA and so the same method was avoided for amplification of test and reference DNA. Similarly, the optimised protocol from the TRL group uses GenomePlex as the WGA method for test and reference DNA (Chris Jones, personal communication). However, the first published paper on single cell a-CGH used DOP-PCR both for target DNA and for test DNA (Hu et al., 2004).

A controversial aspect related to WGA has to do with the reference sample. Different groups have reported reference DNA being amplified with the same method as the single cell (Hellani et al., 2004) or gDNA amplified with the same method (Fuhrmann et al., 2008) or gDNA without amplification (Fiegler et al., 2007). The idea that reference DNA should be similar to the test DNA is based on the fact that the artefacts produced during WGA would be minimised. A very well presented paper regarding WGA on gDNA from small quantities showed how background noise increased when reference DNA was from different the test DNA (Knijnenburg et al., 2007). Two different groups with results on single cell a-CGH concluded that the SD was decreased when gDNA was used as reference. In this project, in the one experiment (experiment C4) gDNA was used as reference but due to the very high SD obtained, in the following experiments similar DNA to test sample was used. A direct comparison to see if reference DNA would make a difference in the result was not obtained. However, by comparing this result with single blastomere a-CGH experiments SD was again very high.

#### **4.7.3 Analysis method and maximum resolution**

Due to the high SD, analysis could not be based on results from individual clones but rather on the clustering and normalising data from many spots in order to detect gain or loss at a specific region. This was achieved with the use of a different algorithm provided by BlueGnome, which diminished the resolution to whole chromosome changes. In every single cell a-CGH published paper some kind of clustering of results has been done which resulted in minimising the resolution of the array experiment. Fuhrmann et al. (2008) reported high resolution (4.4 MB) analysis but further analysis with qPCR showed that these were not single chromosome changes (4-7 copies). Another group claimed that

SNP array analysis on MDA products from single cells could be similar to gDNA results with a 2-3MB smoothing (Iwamoto et al., 2007). In addition, Fiegler et al. (2007) claimed to have identified a previously unknown 8.3MB deletion from a stable cell line even though no confirmation of the result was performed. Finally, Le Caignec et al. (2006) reported a maximum 34MB resolution, but this abnormality was already known and the analysis focused separately in this region of the genome. The same group reported that if 60 clones in a row were consequently amplified or deleted a chromosomal imbalance would be detected without previous knowledge. With the use of 1MB array slides though, this would mean a resolution of 60MB which is bigger than the whole of chromosome 21. Generally, as discussed by Wells et al. (2008) single cell a-CGH work may only be reliable for whole chromosome changes. With this respect, the prenatal slides would be more useful and cheaper for PGS.

The results obtained from this project were analysed with two ways. First the analysis was performed with BlueFuse Cytochip analysis and secondly with the use of the prenatal algorithm. With BlueFuse Cytochip analysis most of the clones appeared as amplified or deleted due to the high SD. The analysis could only be done by looking at the ideograms of each chromosome separately and making assumptions about the ploidy according to if the majority of the clones were above or below zero of log<sub>2</sub> intensity ratio. As a robust and clear result cannot be based on assumptions, the use of the prenatal algorithm was used for the blastomeres where the karyotype was unknown. For the experiments C6-C9 with normal lymphocytes the only shift in the majority of the clones was observed for chromosomes X and Y. For blastomere D1, both Cytochip ideograms and analysis with prenatal algorithm showed the same result, which was a male normal blastomere. For experiments D2 and D4 however, the results obtained from the two analysis methods were different. In D2, analysis with ideograms showed an amplification of chromosomes 5 and Y. Analysis with the prenatal algorithm confirmed amplification on chromosome 5 but not for chromosome Y. Molecular analysis showed that the blastomere was female unless ADO was present but did not confirm the trisomy on chromosome 5. Experiment D3 was not considered reliable as the majority of the clones were excluded from analysis. Finally, Cytochip analysis of experiment D4 created

suspicious for amplification of chromosomes 1, 19 and Y and deletion for chromosomes 8, 13, 14 and 16. The prenatal algorithm only confirmed the amplification of chromosome 19 although more chromosomal imbalances were expected as the embryo was characterised as chaotic. Molecular analysis also confirmed amplification of chromosome 19 but also showed the presence of chromosome Y in the test sample. Ideally, more markers should be used in order to confirm the blastomeres a-CGH result but it was clear that the prenatal algorithm failed to show the correct karyotype of the blastomere.

#### **4.7.4 Conclusion**

Overall, the results obtained from this project showed how difficult it is to analyse single cells by a-CGH. The experiments with single lymphocytes and the Cytochip prenatal slides managed to show better results with less false positives regardless of the WGA method used. Generally, all the slides apart from BlueGnome Cytochip 1MB and prenatal failed to show any reliable result. The single lymphocytes with Cytochip 1MB (experiment C6) showed that even such a noisy result could be analysed when the prenatal algorithm was used. In the single blastomere a-CGH experiments, the karyotype was unknown and more markers on different chromosomes should have been used in order to confirm the results. The prenatal algorithm this time was shown to have a misdiagnosis on at least one chromosome (chromosome Y, experiment D4). If more markers were used on the MDA product for chromosomes 1, 8, 13, 14 and 16 a more reliable outcome could have been made. The aims of this project were not met entirely as more results will be needed for optimisation of the technique. However, the fact that sex mismatch was detected with the Cytochip 1MB and prenatal slide is promising for future development of single cell a-CGH. From the literature it is clear that it is only matter of time until reliable single cell a-CGH will be applied widely for research and diagnostic purposes. The first clinical PGS a-CGH case has already been reported (Hellani et al., 2008). The key to the success will be a combination of a highly reliable and optimised array platform for single cells (Furhmann et al., 2008) and an optimised WGA method for

single cells. As shown by Iwamoto et al. (2007) the a-CGH result was largely dependent on the quality of amplification products.

## **Chapter 5**

### **A-CGH analysis of coelomic fluid**

#### **5.1 Hypothesis**

The hypothesis of this chapter is that aneuploidy can be detected in coelomic fluid using a-CGH.

The DNA collected from coelomic fluid is considered to be degenerate and of low quantity which is one of the biggest limitations for considering coelocentesis as a new technique of prenatal diagnosis. However, if reliable diagnosis by a-CGH from DNA extracted from coelomic fluid can be achieved then this would be a step forward to use coelocentesis as an early prenatal diagnosis technique.

#### **5.2 Introduction**

Coelocentesis has rarely been applied clinically (Makrydimas et al., 2004). The big advantage of this technique is that prenatal diagnosis can be performed very early, from the 5<sup>th</sup> to the 11<sup>th</sup> week of gestation (Jauniaux et al., 2003). The benefit of having an early prenatal diagnosis is that the parents can be reassured much earlier if the fetus is normal. In the case of an affected fetus they can have a safer termination of pregnancy (Makrydimas et al., 2002). Early diagnosis also has the potential for stem-cell therapy to be performed before the 11<sup>th</sup> week where the fetus is immunologically intolerant (Jouannic et al., 2006; Makrydimas et al., 2004). Coelocentesis is not a difficult technique to learn and only induces minimal discomfort to the mother (Jauniaux et al., 2003). As coelocentesis does not require puncture of the placenta or the amniotic sac, the risk of directly injuring the fetus or the placenta is very low (Jauniaux et al., 2003; Jurkovic et al., 1993).

Coelocentesis has also many limitations. One of the most important drawbacks is information on the fetal loss rate. One group reported a 25% (Ross et al., 1997) fetal loss in contrast to the 2% fetal loss reported by a different group (Makrydimas et al., 2002)

even though similar techniques were used. Further investigation has to be done to resolve this issue. Another limitation of coelocentesis is contamination by maternal blood (Jouannic et al., 2006). Many studies have found coelomic fluid samples to be contaminated with maternal DNA which implies that coelocentesis would need further improvement for clinical application (Jauniaux et al., 2003; Jouannic et al., 2006). In order to avoid contamination in the current studies the first 0.2ml of coelomic fluid that is collected are discarded and a new needle and syringe is used for the extraction of the pure sample (Jauniaux et al., 2003).

The most profound problem related to coelocentesis is that the samples collected contain little DNA within poor quality. A recent study has shown that the density of the coelomic fluid cells ranges from zero to ten cells per microlitre which makes a maximum amount of DNA available for diagnosis to be 60ng from 1ml coelomic fluid (Jouannic et al., 2008). FISH experiments have shown that the DNA quality present in the coelomic fluid cells is poor, as most cells are degenerate (George Makrydimas, personal communication). Although up to 4ml of coelomic fluid can be aspirated by coelocentesis there is a suggestion that extracting more than 1ml would increase the risk of fetal loss (Eric Jauniaux, personal communication).

A limited number of studies have been performed on coelomic fluid samples. Jauniaux et al. (2003) published a successful QF-PCR analysis for chromosomal abnormalities of chromosomes X, Y, 18 and 13 but the maternal contamination was found to be in the majority of the samples. In contrast Jouannic et al. (2006) used real-time PCR and obtained a 58% diagnosis when using stringent criteria. FISH analysis of 11 chromosomes on interphase nuclei from coelomic fluid samples has also been reported (Chatzimeletiou et al., 2005).

### **5.3 Aims**

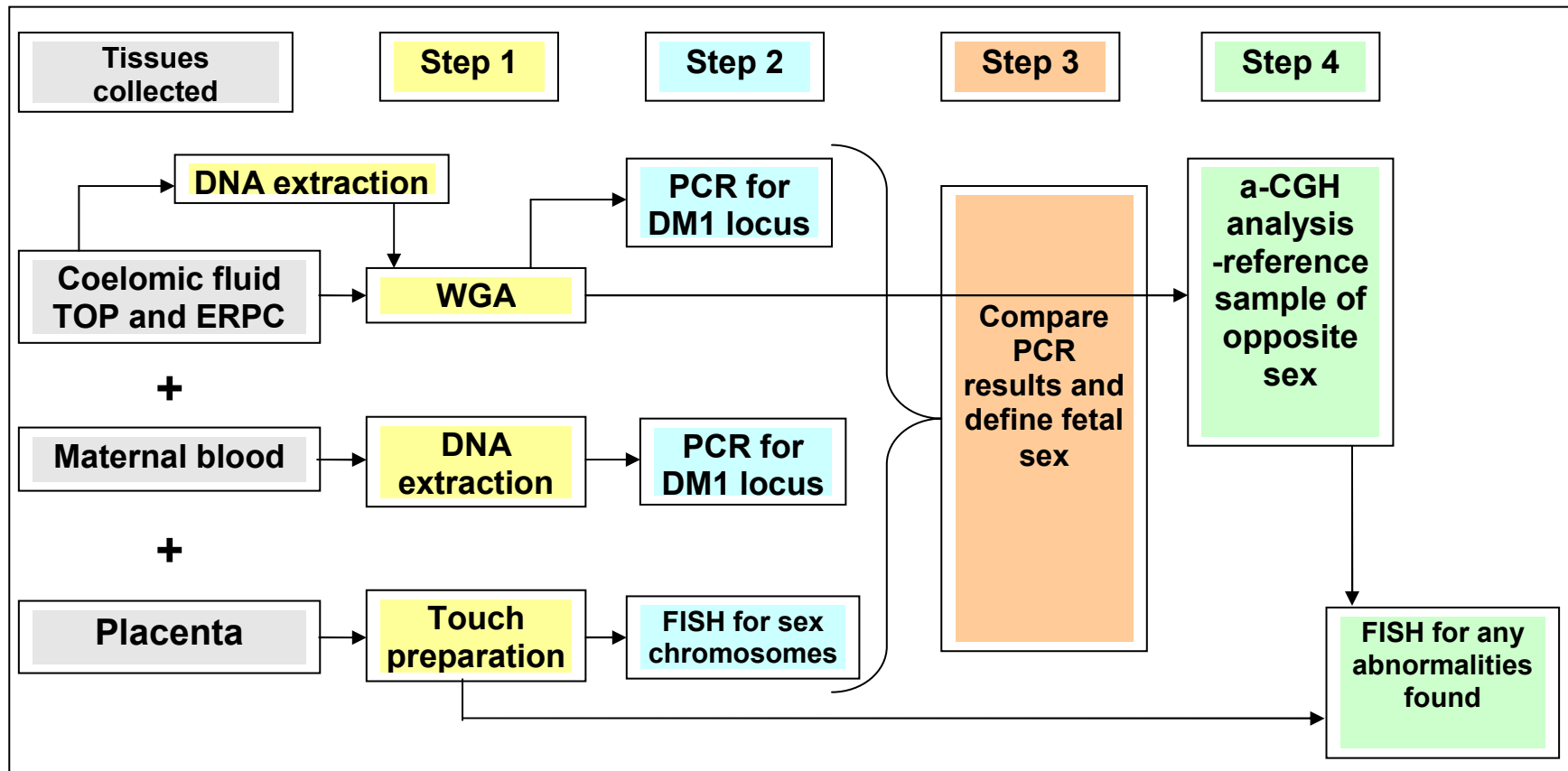
The aim of this project was to perform successful molecular cytogenetic analysis by a-CGH with DNA from coelomic fluid samples. The challenge of this project was the low

quantity and quality of DNA available. For this reason, WGA had to be considered in order to produce reliable and robust results. MDA and GenomePlex were used and compared as WGA techniques for DNA amplification of the coelomic fluid samples. The optimisation of coelomic fluid samples on a-CGH could be beneficial if coelocentesis would ever be considered and applied as an early form of prenatal diagnosis. The application of WGA direct on coelomic fluid without previous DNA extraction was also considered in order to investigate if a minimum amount of coelomic fluid could be extracted for molecular cytogenetic analysis.

For this project, DNA was extracted from coelomic fluid samples, and amplified by MDA or GenomePlex. Coelomic fluid was aspirated from pregnant women that were having termination of pregnancy (TOP) for social reasons or evacuation of retained products of conception (ERPC) in case of miscarriage. Contamination of the sample and sample sex was tested with the use of PCR (DM I triplex) and FISH (sex chromosomes) respectively. A total of 20 a-CGH experiments were performed with five coelomic fluid samples.

#### **5.4 Experimental design**

Coelomic fluid was collected using coelocentesis from women just before they underwent TOP or ERPC. Maternal blood and a small piece of placenta were collected with each coelomic fluid sample. The DNA was extracted from coelomic fluid and MDA or GenomePlex was applied to 5µl of the extracted DNA. In one case MDA was applied directly on 5µl of coelomic fluid, without DNA extraction. The amplified products were used for molecular analysis using the DM triplex locus. The same molecular tests were applied on DNA extracted from the maternal blood and the results were compared in order to check for contamination. FISH was applied on the placenta in order to detect the fetal sex and this was used for sex mismatch experiments on a-CGH. The WGA products from the coelomic fluid samples were applied on a-CGH slides. Any abnormalities detected were confirmed with FISH analysis on the placenta sample. Figure 5.1 summarises the experimental design.



**Figure 5.1: Summary of experimental design for the coelomic fluid study.** This diagram shows how the experiments of this chapter were designed in order to make a valid conclusion. After collection of samples (coelomic fluid, maternal blood and placenta) a series of experiments were done in order to determine which coelomic fluid samples were pure, what was the fetal sex and finally to do a-CGH analysis. Any abnormalities detected were compared with FISH analysis from the placenta samples. TOP: termination of pregnancy; ERPC: evacuation of retained products of conception.



## **5.5 Materials and Methods**

### **5.5.1 Ethical approval**

For this chapter ethical approval was obtained from the University College London Hospitals Committee on the Ethics of Human Research and all women gave written consent after receiving complete information about the procedure.

### **5.5.2 Coelocentesis and tissues collection**

Coelocentesis was performed by Professor Eric Jauniaux in University College London Hospital. The procedure took place immediately before TOP or ERPC under general anaesthesia. The gestational age was determined by ultrasound measurement of the crown–rump length. For the coelomic fluid collection, the external genitalia and the vagina were cleaned with an antiseptic solution and coelomic fluid was collected by transvaginal puncture under ultrasound guidance using a 20G needle. The first 0.2-0.4ml of the coelomic fluid were discarded in order to avoid maternal contamination and the next 0.2-3ml were collected with a new 20G needle in 2ml syringes. Together with the coelomic fluid sample, maternal blood was collected for contamination check and also placenta tissue after TOP or ERPC was obtained for confirmation of the results.

### **5.5.3 DNA extraction**

The coelomic fluid was transferred to 1.5ml microcentrifuge tubes, kept on ice and transferred immediately to the main lab for DNA extraction using the Puregene genomic DNA purification kit (Gentra Systems, USA) (Appendix A3). The protocol followed from the kit was one for DNA extraction from 50µl body fluid for low cell numbers. Briefly the coelomic fluid was centrifuged at 8,000rpm for 10 minutes in order to concentrate the samples and the supernatant was discarded leaving a white pellet and ~50µl of fluid. A maximum of 1ml coelomic fluid was used for DNA extraction. Following that, 250µl of Cell lysis solution was mixed well with the CoF, 1.5µl of

proteinase K (20mg/ml) was added and the sample was incubated at 55°C for 1 hour to complete lysis. After that, 1.5µl of RNase A solution (Gentra Systems, USA) was added and the samples were incubated at 37°C for 30 minutes. The samples were cooled on ice for 1 minute and 100µl of protein precipitation solution was added to the lysate. The sample was vortexed for 20 minutes, incubated on ice for 5 minutes and centrifuged at full speed for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5ml tube while the protein-pellet content was discarded. For DNA precipitation, 300µl of 100% isopropanol and 0.5µl of glycogen solution (Gentra systems, USA) was mixed gently with the DNA and incubated at room temperature for 5 minutes. The sample was centrifuged at full speed, the supernatant was discarded and the DNA was washed in 300µl of 70% ethanol. The DNA was centrifuged again at full speed, the supernatant was discarded and the DNA was left to air dry for 5 minutes. The DNA was hydrated in 20µl DNA hydration solution at 65°C for 1 hour and stored at -20°C. For DNA extraction of blood, the protocol was as described in section 2.2.1.

#### **5.5.4 WGA, PCR and DNA concentrations**

WGA for a-CGH analysis and PCR using the DM I locus was performed with MDA or GenomePlex. WGA methods and protocols are described in section 2.4. Protocols P3 (MDA on gDNA) and P5 (WGA2, GenomePlex on gDNA) were applied to the reference samples and the DNA extracted from the coelomic fluid samples whereas protocol P4 (MDA on coelomic fluid) was applied directly to coelomic fluid without previous DNA extraction. The triplex DM 1 PCR reaction was applied to DNA extracted from maternal blood and MDA products from the coelomic fluid samples. The DM 1 protocol was described in section 3.5.4.1. Measuring the DNA concentration was performed only for the WGA products that were used for a-CGH experiments after sample purification. DNA purification and concentration method are described in section 4.5.3.

### 5.5.5 DNA and tissue materials collected

All the DNA and the tissue materials collected for this project are summarised in table 5.1. The table includes the coelomic fluid reference from TOP and ERPC samples collected, the fetus age, the amount of coelomic fluid collected, the collection of maternal blood and placenta tissue and the method of WGA used to amplify the coelomic fluid.

CoF sample reference	Weeks of pregnancy	Amount of coelomic fluid Collected	Maternal blood and Placenta collected	Method of WGA applied to coelomic fluid
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#### TOP samples

CoF1	9W 5D	1ml	✓	MDA
CoF2	9W	2ml	✓	MDA
CoF3	9W 2D	4ml	✓	MDA, GenomePlex
CoF4	9W 4D	4ml	✓	MDA, GenomePlex
CoF5	10W 5D	2ml	X	MDA
CoF6	8W 3D	2ml	✓	MDA
CoF7	6W 5D	1ml	✓	MDA, GenomePlex
CoF8	6W 4D	1ml	✓	MDA, GenomePlex
CoF9	9W	1.5ml	✓	MDA

#### ERPC samples

CoF10	11W 3D	2ml	Maternal blood only	MDA, MDA direct of CF, GenomePlex
CoF11	8W	2ml	✓	MDA, GenomePlex
CoF12	8W	2ml	✓	MDA
CoF13	9W	4.5ml	✓	MDA

**Table 5.1: Details of the coelomic fluid and other materials collected.** This table summarises all DNA and tissue materials collected for this project. Sign “✓” is used to show that maternal blood and placenta were collected and sign “X” is used to show that maternal blood and placenta were not collected or WGA was not performed.

## 5.5.6 Fluorescent in Situ Hybridisation

### 5.5.6.1 Touch preparation

FISH experiments were applied on placental tissue in order to obtain the fetal gender and to confirm results from a-CGH analysis. Placental tissue was collected after TOP or ERPC from women that also provided the coelomic fluid. The placenta samples were either used directly or were frozen at -80°C for up to two weeks prior to touch preparations. The placenta was washed three times in Hank's media (HBSS) on a Petri dish and any non-embryonic material was removed with the use of a scalpel in order to avoid contamination. The slides were prepared by washing first in ethanol for 5 minutes, then in methanol for 10 minutes and finally in fix solution (3 volumes of methanol in 1 volume of acetic acid) for 30 minutes. After the slides were dried, a small piece of tissue was dapped and spread along each slide. Seven slides were produced from each placental tissue and were stored at 4°C.

### 5.5.6.2 Probe preparation

The probe mix consisted of probes for up to three chromosomes and buffer (CEP buffer for centromeric probes and LSI for locus specific probes). The total volume was always 5µl per sample. Table 5.2 shows all volumes for probe mixes.

Probe mix	Amount	Buffer
X(SG)/Y(SR)/18(SA)	2.5µl	CEP: 2.5µl
13(SG)/21(SO)/15(SA)	13/21:0.6µl/ 15:0.5µl	LSI: 2.5µl

**Table 5.2: Summary of probe mixes used for the prenatal project.** One or two rounds of FISH experiments were conducted with each slide after touch preparations. SG: spectrum green, SR: spectrum red and SA: spectrum aqua.

### **5.5.6.3 FISH methods**

All the FISH methods regarding denaturation (co- and separate), hybridization and post-hybridisation washes and image analysis interpretation have been described in sections 4.5.6

### **5.5.7 Molecular cytogenetic analysis by a-CGH**

Molecular cytogenetic analysis by a-CGH was performed for the analysis of the coelomic fluid samples. Slides from BlueGnome (prenatal slides and 1MB slides), the TRL Mermaid 32K array slides and Spectral Genomics constitutional slides were used for this project. As reference material, WGA products amplified in the same method as the test samples and opposite sex were used. The initial DNA that was used for WGA was 10ng. The methods for a-CGH, labelling, hybridization, scanning and analysis are described in detail in section 4.5.4. All the experiments apart from the ones with the 32K mermaid slide were performed in dye swap.

## **5.6 Results**

### **5.6.1 Molecular analysis for contamination**

The first experiments focused on distinguishing which samples were contaminated with maternal blood and which were pure. As coelocentesis is not yet a validated technique and the amount of coelomic fluid cells is very limited, even a small amount of contamination could have a great effect on the outcome of the experiment. Only pure samples would be analysed by a-CGH. Coelomic fluid has a clear yellowish colour. The transparency is usually lost when the fluid is contaminated with blood and the colour may become light or dark yellow. The blood could be either from the mother or the fetus. Blood contamination was also physically observed during the DNA extraction protocol,

in the first step that the samples were centrifuged for 10 minutes at 6,000 rpm. After centrifugation the coelomic fluid cells were usually observed as a small tiny white pellet. Sometimes the pellet was not seen probably due to the very small amount of cells present. A red spot was also present in all the samples when the colour was not clear and this confirmed the blood contamination of the coelomic fluid samples.

In order to determine if the samples were contaminated by maternal blood, a contamination check was performed using PCR analysis of three STR markers on chromosome 19 (DM triplex reaction). PCR analysis was performed on the MDA products and the results were compared with the alleles obtained from the maternal DNA after DNA extraction from blood. Although it could not be proved which samples were contaminated as the paternal DNA was not available, it was easy to find samples that were free of contamination. Pure samples were considered in samples where one allele was identical to the mother and a second that was not the same. The results are shown on table 5.3.

From the results presented in this table it was shown that TOP samples CoF 3, CoF 4, CoF 7, CoF 8 and ERPC sample CoF 10 had a clear colour. CoF 9 sample did not show any results which could be because of MDA failure or because no cells were available. CoF 11 sample was considered either contaminated or the fetus had trisomy 19 as three alleles were present at two loci. For CoF 10 where the MDA product was derived directly from 5µl of coelomic fluid without DNA extraction, PCR analysis revealed identical alleles with the CoF 10 MDA sample (result not presented on table 5.3). In samples CoF 1, CoF 2, CoF 5, CoF 6, CoF 12 and CoF 13 the PCR alleles were identical to the maternal DNA and were excluded from further experiments because it could not be concluded if they were contaminated or not.

Samples	APOC2	DM I triplex DM	D19S112	CoF Colour
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***TOP samples***

<b>CoF 1</b> MDA sample	139 - 150	140 - 146	114 - 122	<i>Light Yellow</i>
CoF 1 Maternal DNA	139 - 150	140 - 146	114 - 122	
<b>CoF 2</b> MDA sample	135 - 150 - 155	122 – 140	117	<i>Light Yellow</i>
CoF 2 Maternal DNA	135 - 150	122 – 140	117 - 123	
<b>CoF 3</b> MDA sample	135 - 152	138 - 145	128	<i>Clear</i>
CoF 3 Maternal DNA	135 - 154	138 - 145	120 - 128	
<b>CoF 4</b> MDA sample	155 - 161	122 -149	130 - 133	<i>Clear</i>
CoF 4 Maternal DNA	151 - 161	122 - 131	133	
<b>CoF 5</b> MDA sample	152 - 154	122 - 140 -171	121 - 133	<i>Light Yellow</i>
CoF 5 Maternal DNA	152 - 154	140 – 171	121 - 133	
<b>CoF 6</b> MDA sample	129 - 150	144 - 151	123 - 136	<i>Dark Yellow</i>
CoF 6 Maternal DNA	129 - 150	144 - 151	123 - 136	
<b>CoF 7</b> MDA sample	152 - 155	133 - 139	128 - 135	<i>Clear</i>
CoF 7 Maternal DNA	155	122 - 139	128 - 135	
<b>CoF 8</b> MDA sample	148 - 155	119 - 132	120 - 130	<i>Clear</i>
CoF 8 Maternal DNA	148 - 152	119	120 - 141	
<b>CoF 9</b> MDA sample	-	-	-	<i>Clear</i>
CoF 9 Maternal DNA	135 - 150	146 - 154	117	

***ERPC samples***

<b>CoF 10</b> MDA sample	151 - 156	123 - 170	117 - 133	<i>Clear</i>
CoF 10 Maternal DNA	156	123	129 - 133	
<b>CoF 11</b> MDA sample	139 - 151	140 - 150 - 154	117 – 122 - 128	<i>Light Yellow</i>
CoF 11 Maternal DNA	135 – 139 - 151	140 - 154	117 - 122	
<b>CoF 12</b> MDA sample	150	140 - 155	122 - 133	<i>Dark Yellow</i>
CoF 12 Maternal DNA	150	140 - 155	122 - 133	
<b>CoF 13</b> MDA sample	148 – 154	122 - 180	123	<i>Dark Yellow</i>
CoF 13 Maternal DNA	148 – 154	122 - 180	123	

***Table 5.3: Allele sizes of CoF samples and maternal DNA.*** The table summarises the PCR results of the three loci of the DM triplex reaction from all the MDA products of CoF DNA and the maternal DNA. The CoF colour shows the colour of the samples prior to DNA extraction.

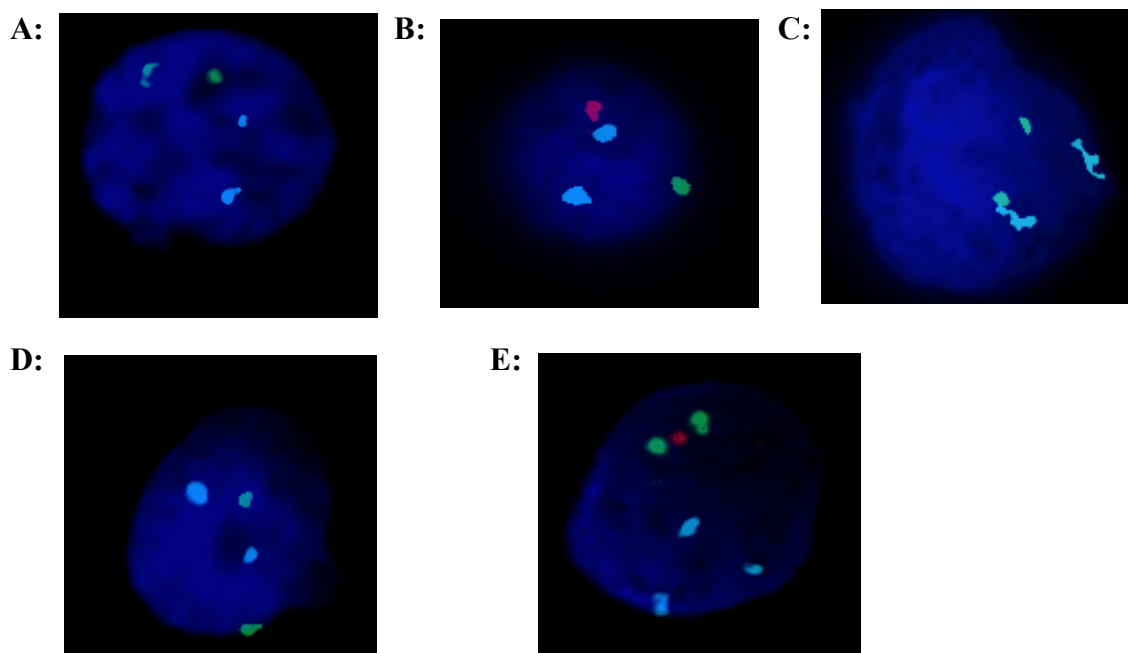
### 5.6.2 FISH analysis

The gender determination of the fetus was necessary in order to conduct sex mismatch experiments with a-CGH. The probe mix contained probes for chromosome X, Y and 18. The results are shown on Table 5.4 and Figure 5.2. FISH experiments were not done for CoF 10 because a karyotype had already been performed which revealed that the embryo was a male with trisomy 21 (47, XY + 21). For CoF 11 two X chromosomes, one Y chromosome and three 18 chromosomes were identified. These findings, together with the molecular analysis on chromosome 19 increased the suspicions that the sample was triploid (69, XXY).

Placenta tissue samples of	Fluorescent signals			Result
	X	Y	18	
CoF3	2	0	2	Female
CoF4	1	1	2	Male
CoF7	2	0	2	Female
CoF8	2	0	2	Female
CoF11	2	1	3	Male

**Table 5.4: Fluorescent signals for chromosomes X, Y and 18 from the placenta tissue samples:** This table summarises the number of fluorescent signals obtained after FISH analysis on the placenta tissue samples so that the sex of the fetus could be determined.





**Figure 5.2: FISH analysis of placenta samples for chromosomes 18, X and Y.** These figures show the results of the placenta samples obtained in order to determine the gender of the fetus. A: placenta of CoF 3, B: placenta of CoF 4, C: placenta of CoF 7, D: placenta of CoF 8 and E: placenta of CoF 11. From FISH analysis it was concluded that samples CoF 3, CoF 7 and CoF 8 were female whereas CoF 4 was male. CoF 11 was chromosomally abnormal as three chromosomes 18, two chromosomes X and one chromosome Y was detected. Spectrum aqua: chromosome 18, Spectrum Green: chromosome X and Spectrum red: Chromosome Y.

### 5.6.3 DNA concentration

The DNA concentrations of the coelomic fluids and the WGA products are listed in table 5.5. The literature (Jouannic et al., 2008) reports that zero to ten fetal cells are present per microlitre of coelomic fluid which means that the maximum DNA that could be obtained from a DNA extraction would be 60ng of DNA (6pg of DNA per cell x 10,000 cells maximum present in 1ml of coelomic fluid). Since the DNA extracted from the coelomic fluid was diluted in 20µl of hydration solution the maximum concentration expected from was 1.5ng/µl. However, from the results obtained, the DNA concentration of coelomic fluid was much more than what was expected. DNA from cell free DNA (cfDNA) that is

present in CoF could be the reason for increasing the final DNA yield. Similar results have been found by Jouannic et al. (2008). Nevertheless, it was very likely that measuring DNA concentration at such low quantities could produce incorrect results. Measuring DNA concentration by running a gel could provide more accurate results but this was not ideal due to the limited DNA that was available. In one case MDA was also applied to half the amount of DNA (2.5µl instead of 5µl) extracted from the CoF 10.

The DNA concentration from the MDA and GenomePlex products was measured after DNA purification. The optical density (OD) was scored to show the DNA purity. Due to the limited number of a-CGH experiments that could be conducted because of the high costs of the technique five samples were used for a-CGH analysis. CoF 3 sample was excluded from the analysis mainly due to the poor amplification by MDA.

Sample	gDNA		MDA				GenomePlex	
			Amplification 1		Amplification 2			
	ng/µl	OD	ng/µl	OD	ng/µl	OD	ng/µl	OD
CoF 3	13	1.82	32	1.24	-	-	85	1.97
CoF 4	11	1.44	130	1.81	-	-	108	1.92
CoF 7	16	1.26	190	1.8	-	-	328	1.96
CoF 8	9	1.71	100	1.81	-	-	184	1.90
CoF 10	6	1.68	83	1.82	98	1.89	33	1.72
CoF 10 cells	-	-	89	1.77	-	-	-	-
CoF 11	21	1.8	122	1.78	-	-	66	1.68
control male	20	1.80	462	1.80	-	-	527	1.89
control female	20	1.80	458	1.84	-	-	475	1.83

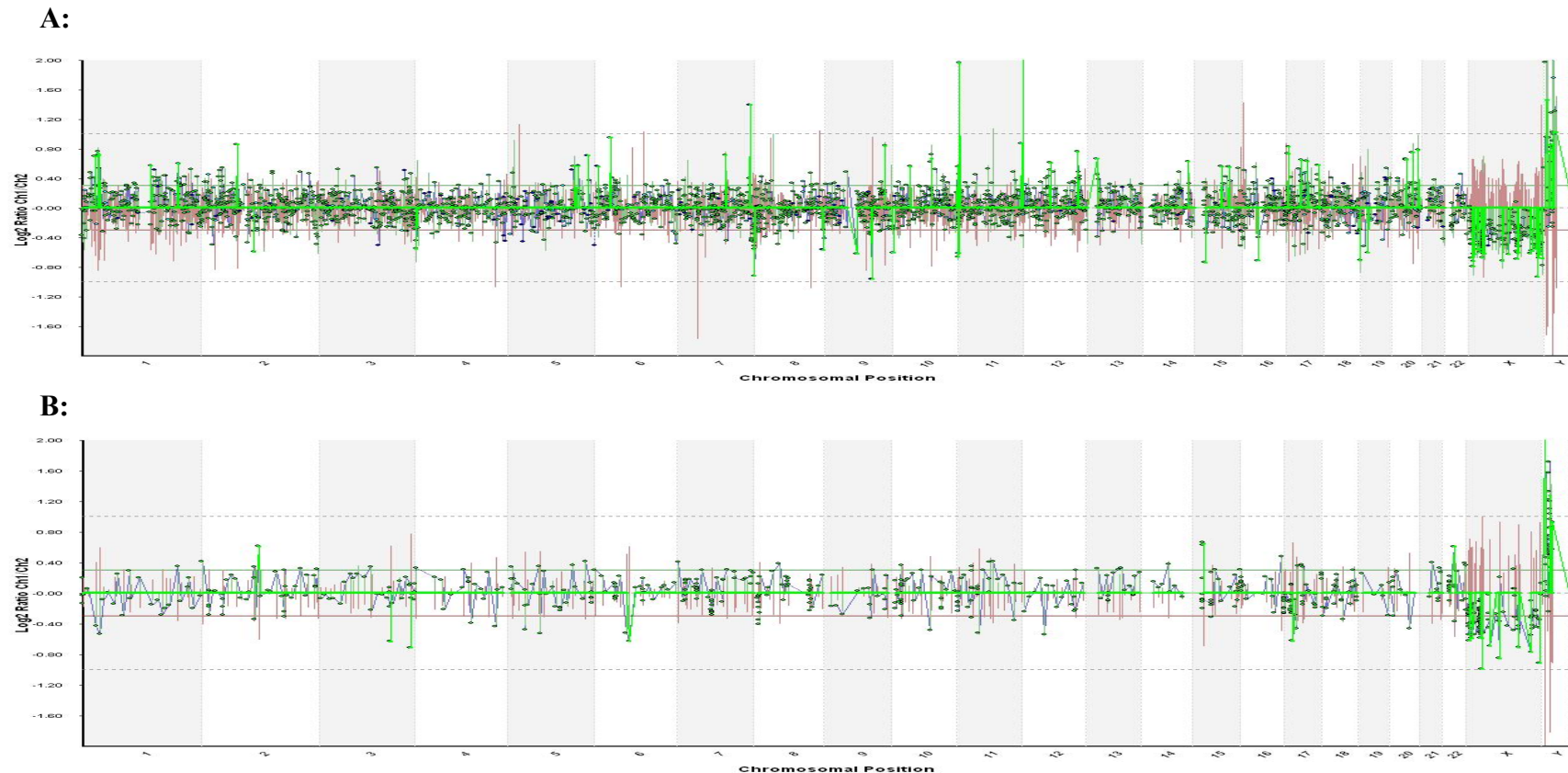
**Table 5.5: DNA concentration of CoF samples and their WGA products.** This table summarises the results from the CoF samples after DNA extraction and after WGA (MDA and GenomePlex). In CoF 10, MDA amplification was performed twice and also direct amplification of the same sample without DNA extraction was performed. OD: optical density.

#### **5.6.4 A-CGH analysis**

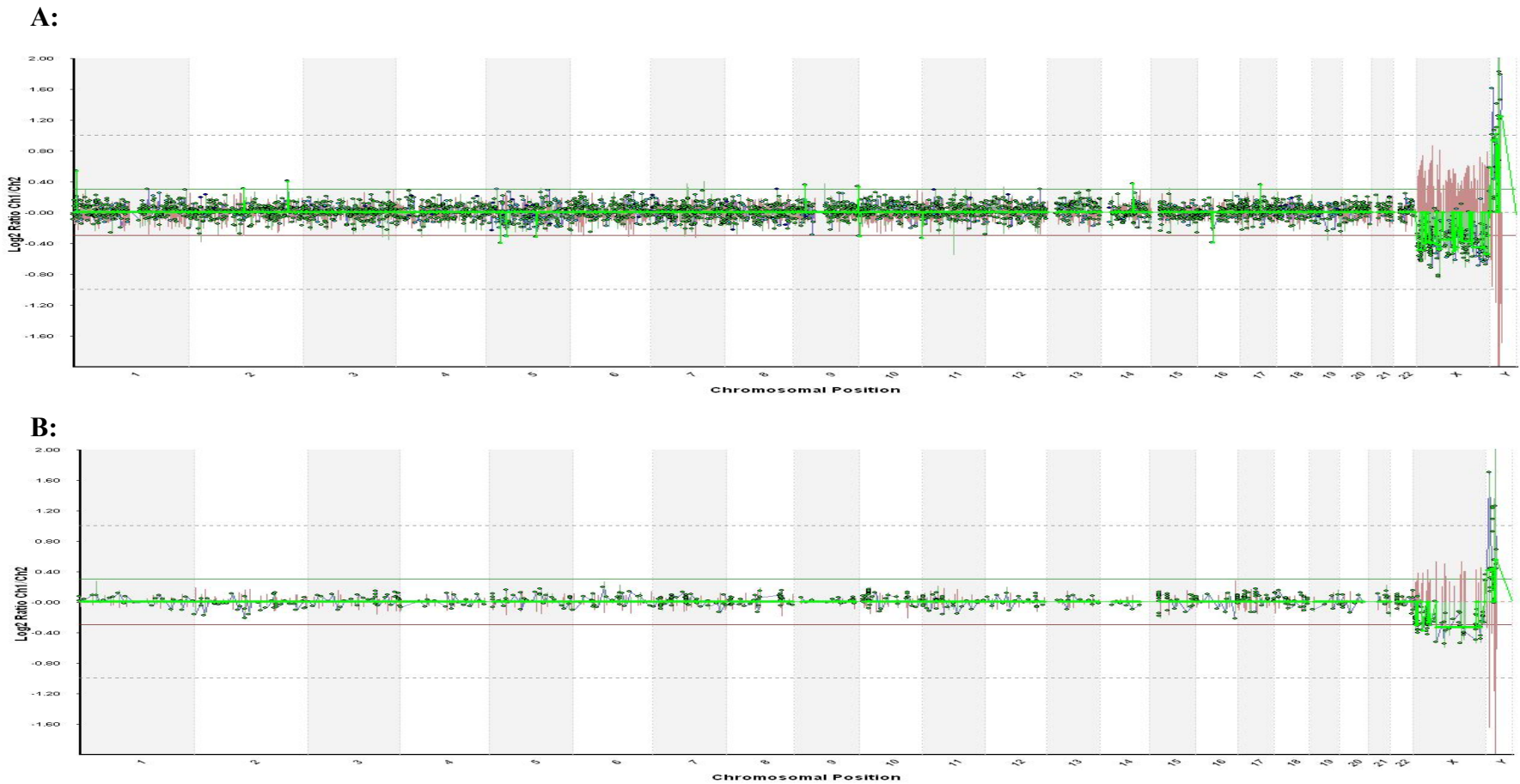
For the a-CGH experiments BAC a-CGH slides of different resolution (10MB, 1MB and 100KB) from different companies (BlueGnome, TRL and Spectral Genomics) were used. For the TOP samples the termination was done for psycho-social purposes and so there was no previous indication for chromosomal imbalances to be present. Thus, the experiments were mainly focused on which slides and amplification method would provide the best results.

##### **5.6.4.1 A-CGH analysis of CoF 4**

CoF 4 DNA was amplified with MDA and GenomePlex. The GenomePlex sample was applied to a Cytochip 1MB and a Cytochip prenatal slide. The MDA product was applied to a Cytochip 1MB, a Cytochip prenatal slide and a TRL 32K Mermaid array slide. The results from the Cytochip arrays are shown on figure 5.3 for the MDA products and figure 5.4 for the GenomePlex products. For the experiments the control DNA used was normal female amplified with a similar method to the test DNA. A-CGH analysis was not performed with the mermaid 32K array due to very poor hybridisation (21.4% probe inclusion). From the results obtained, it was easy to detect sex mismatch in all four experiments but the MDA samples produced much noisier results compared to the GenomePlex products (table 5.6). The GenomePlex CoF 4 - Cytochip prenatal slide experiment was the only one where no single spot exceeded the theoretical log<sub>2</sub> threshold ratio for amplification or deletion apart from the sex chromosomes. In contrast, MDA samples produced many artefacts especially in regions close to the chromosome ends which minimised the resolution analysis to whole/large chromosome changes.



**Figure 5.3: A-CGH analysis of CoF 4 MDA products.** These graphs compare  $\alpha$ -CGH results from two identical test and reference MDA samples applied in high and low resolution slides from BlueGnome. A: CoF 4 MDA sample – Cytochip 1MB, B: CoF 4 MDA sample- Cytochip prenatal slide. Although a large number of individual clones show false positive results, it is obvious that only the majority of the clones for chromosomes X and Y are seen as deleted and amplified respectively which certifies the sex imbalance of test/reference DNA.



**Figure 5.4: A-CGH analysis of CoF 4 GenomePlex products.** These graphs compare a-CGH results from two identical test and reference samples amplified with GenomePlex with high and low resolution slides from the same company A: CoF 4 GenomePlex sample – Cytochip 1MB, B: CoF 4 GenomePlex sample- Cytochip prenatal slide. The results are much clearer compared to the MDA samples and sex mismatch can easily be detected on both arrays. No false positives were detected with the Cytochip prenatal array.

	CoF 4 MDA		CoF 4 GenomePlex	
	Cytochip 1MB	Cytochip prenatal	Cytochip 1MB	Cytochip prenatal
SD for autosomes	0.212	0.203	0.094	0.070
SD for chromosome X	0.179	0.220	0.168	0.126
SD for chromosome Y	0.927	0.552	0.731	0.497
Median between replicates	0.08	0.08	0.08	0.07
Clones included (%)	81.26	98.87	94.20	100

**Table 5.6: Summary of results of the a-CGH with CoF 4.** The table presents useful analysis measurements of the CoF 4 a-CGH experiments. CoF 4 GenomePlex produces much lower SD compared to MDA products.

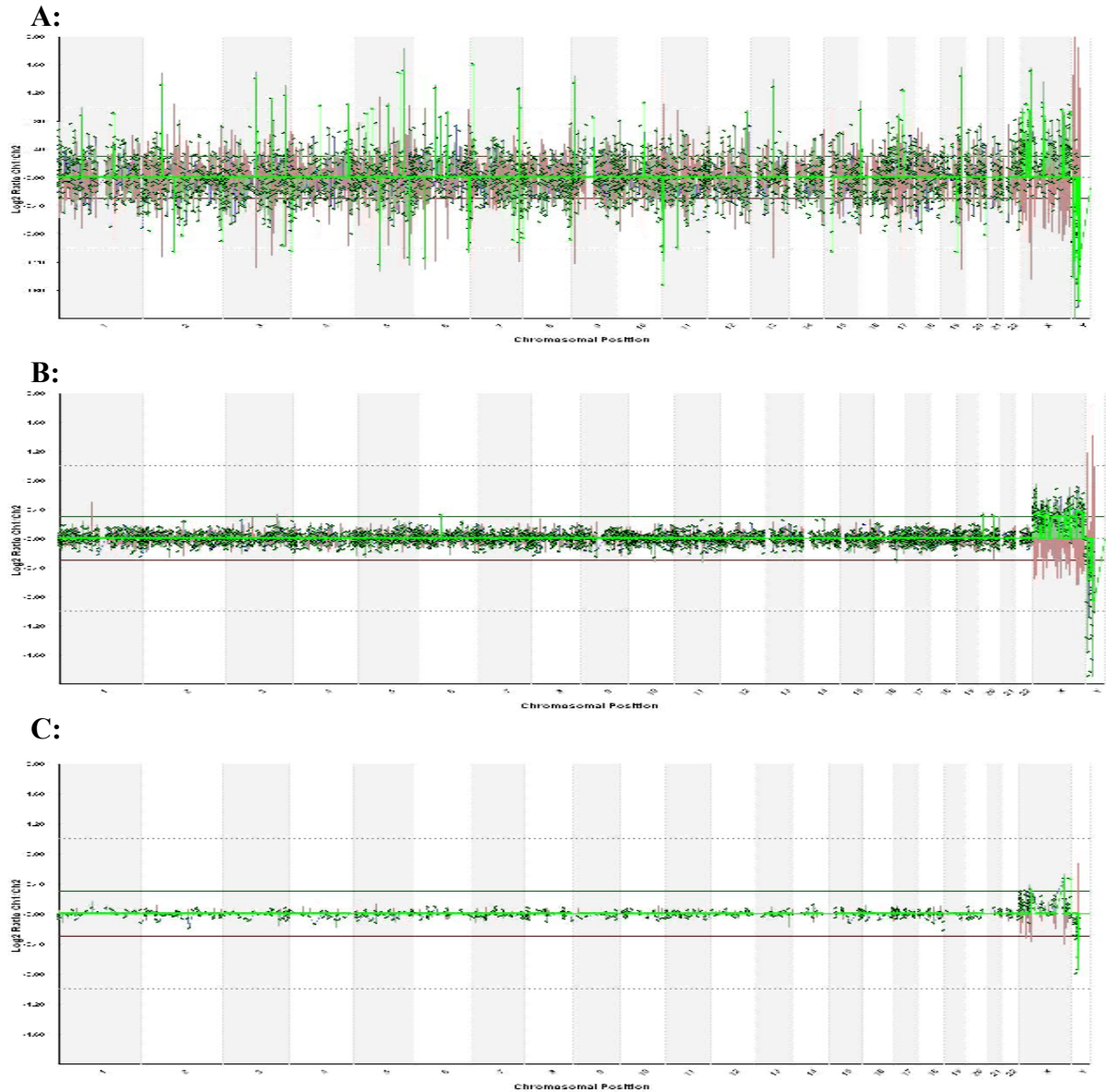
#### 5.6.4.2 A-CGH analysis of CoF 7

Three experiments were conducted with CoF 7. A CoF 7 MDA sample was hybridised against a normal MDA male sample on a Cytochip 1MB slide. Two a-CGH experiments were conducted with GenomePlex products from CoF 7. One Cytochip prenatal and one Cytochip 1MB slides was used for the experiments. Reference sample was normal male amplified in the same way. The results are presented in table 5.7 and figure 5.5. The MDA sample produced very noisy results compared to the GenomePlex products. The CoF 7 GenomePlex sample produced results with very small SD even though a clear difference on the X chromosome was not as clear as in the 1MB slide (figure 5.6).

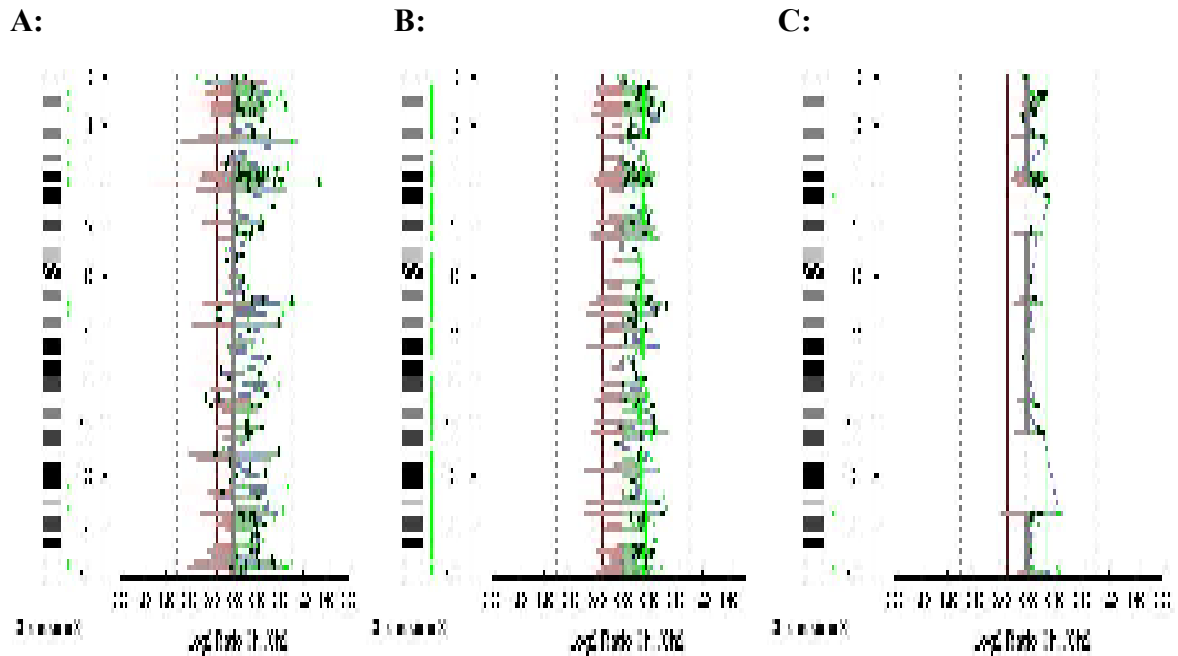
	CoF 7 MDA	CoF 7 GenomePlex	
	Cytochip 1MB	Cytochip 1MB	Cytochip prenatal
SD for autosomes	0.297	0.073	0.043
SD for chromosome X	0.313	0.144	0.096
SD for chromosome Y	0.662	0.501	0.180
Median between replicates	0.07	0.07	0.07
Clones included (%)	95.33	95.57	98.22

**Table 5.7: Summary of results of the a-CGH with CoF 7.** The table presents useful analysis measurements of the CoF 7 a-CGH experiments. The Cytochip prenatal -CoF 7 GenomePlex experiment was the only that did not produce false positive results.





**Figure 5.5:** *A-CGH analysis of CoF 7 MDA and GenomePlex products. The ideograms present the a-CGH results of CoF 7 WGA products with Cytochip 1MB and prenatal slides. A: CoF 7 MDA sample – Cytochip 1MB, B: CoF 7 GenomePlex sample- Cytochip 1MB slide and C: CoF 7 GenomePlex sample- Cytochip prenatal slide. The reduction of noise is clear in the GenomePlex products. In all a-CGH experiments sex mismatch could be identified but with the MDA sample, analysis with the prenatal algorithm would be needed for better identification of the imbalance. For the Cytochip prenatal array, lowering the threshold for amplification would be necessary for the identification of the chromosome X amplification.*

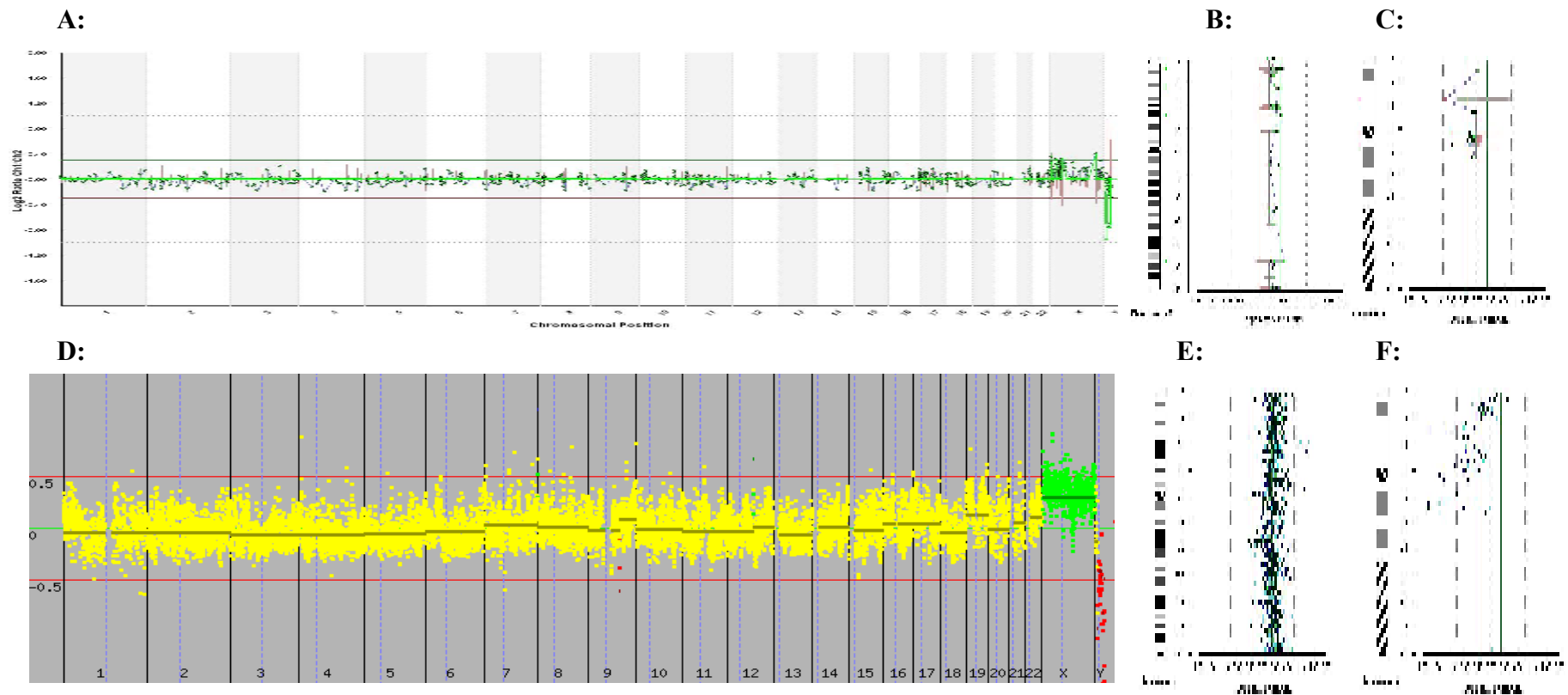


**Figure 5.6: Ideograms of Chromosome X from a-CGH experiments with CoF 7.** Detailed ideograms of chromosome X from a-CGH experiments on CoF 7 (from figure 5.5). A: MDA sample -1MB array, B: GenomePlex sample – 1MB array and C: GenomePlex sample – prenatal array. Although a clear amplification is detected in the 1MB slides in both MDA and GenomePlex products, an imbalance is not seen in the centromeric region on the prenatal slide.

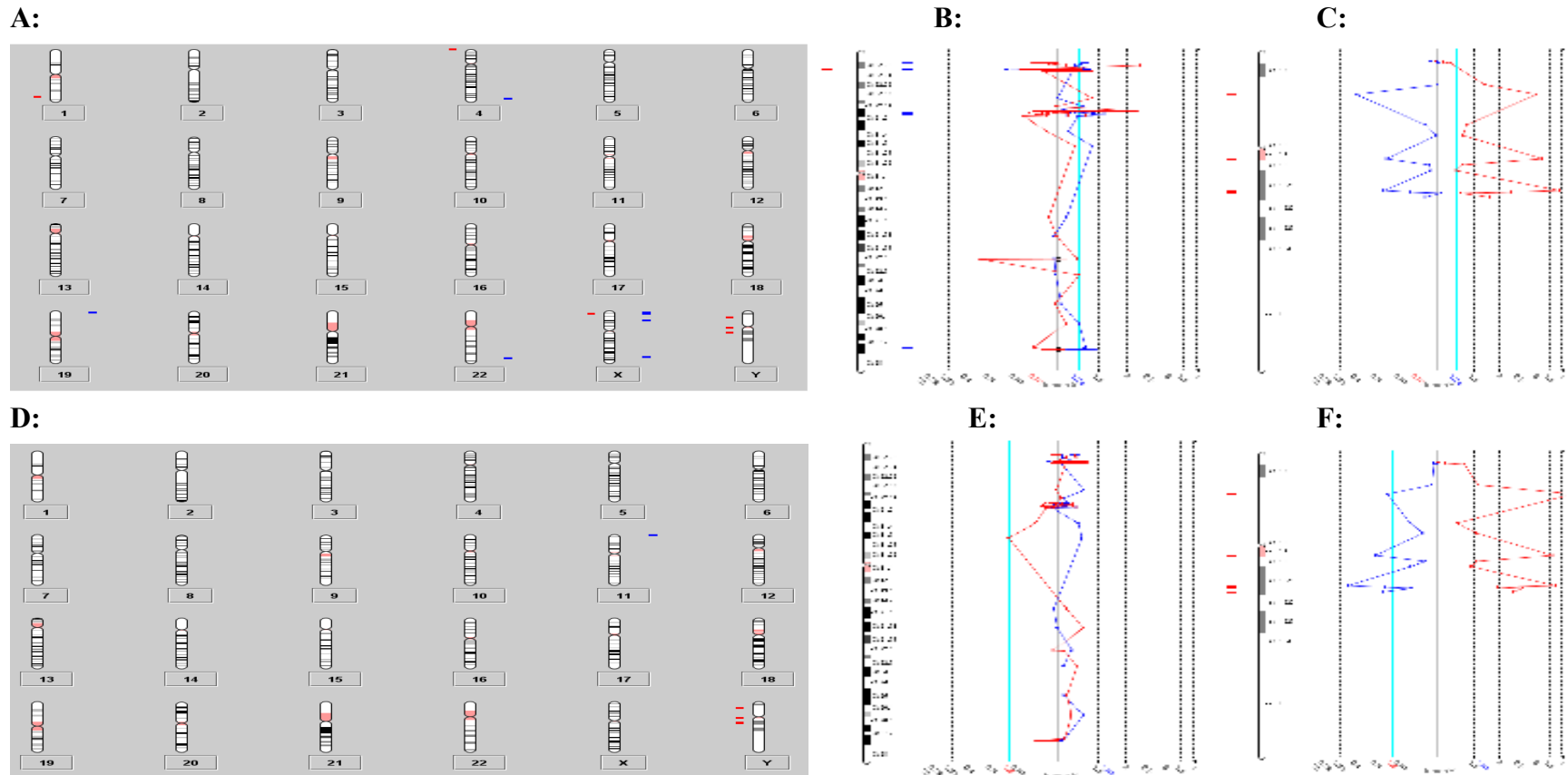
#### 5.6.4.3 A-CGH analysis of CoF 8

Four experiments were conducted with CoF 8. Figure 5.7 compares the results from the first two experiments. DNA of CoF 8 was amplified with GenomePlex and hybridised in two array slides; a Cytochip prenatal slide and a 32K Mermaid array slide. Table 5.8 summarises the analysis data. In the other two experiments a CoF 8 GenomePlex and CoF 8 MDA products were applied on two Spectral Genomics constitutional slides (figure 5.8). Unfortunately, no data were obtained with SpectralWare™ analysis software. The reference sample was a normal male sample amplified in a similar way to the test samples.





**Figure 5.7:** *A-CGH analysis of CoF 8 GenomePlex products with Cytochip prenatal and Mermaid 32K slides. The ideograms compare the a-CGH results of CoF 8 GenomePlex products with Cytochip prenatal slide and 32K Mermaid slide. A: CoF8 GenomePlex sample – Cytochip prenatal slide, B and C: Detailed ideograms of chromosomes X and Y respectively from the Cytochip prenatal slide. D: CoF 8 GenomePlex sample- 32K Mermaid array; E and F: Detailed ideograms of chromosomes X and Y respectively from the 32K Mermaid slide. The sex mismatch was identified with both arrays even though it was not so strong to exceed the log2 threshold ratios for the amplification of the X chromosome. The reference sample was a GenomePlex normal male sample.*



**Figure 5.8:** A-CGH analysis of CoF 8 GenomePlex and MDA products Spectral Genomics constitutional slides. The ideograms compare the a-CGH results of GenomePlex and MDA CoF 8 products using the Spectral Genomics constitutional slides. A: CoF8 GenomePlex sample – Spectral Genomics Constitutional slide. B and C: Ideograms of chromosomes X and Y respectively from the GenomePlex product. D: CoF 8 MDA sample- Spectral Genomics Constitutional slide; E and F: Ideograms of chromosomes X and Y respectively from the MDA product. Only the experiment with the GenomePlex product identified the chromosome X amplification.

	<b>CoF 8 GenomePlex</b>	
	Cytochip prenatal	Mermaid 32K
SD for autosomes	0.071	0.212
SD for chromosome X	0.095	0.214
SD for chromosome Y	0.220	0.579
Median between replicates	0.07	0.31
Clones included (%)	98.81	67.35

**Table 5.8: Summary of results of the a-CGH with CoF 8.** This table presents the data provided by the analysis of Cytochip prenatal and the Mermaid 32K arrays by BlueFuse software. There is a large difference in these results although the same samples were hybridised. Both arrays detected an Y chromosome deletion and weak amplification of chromosome X.

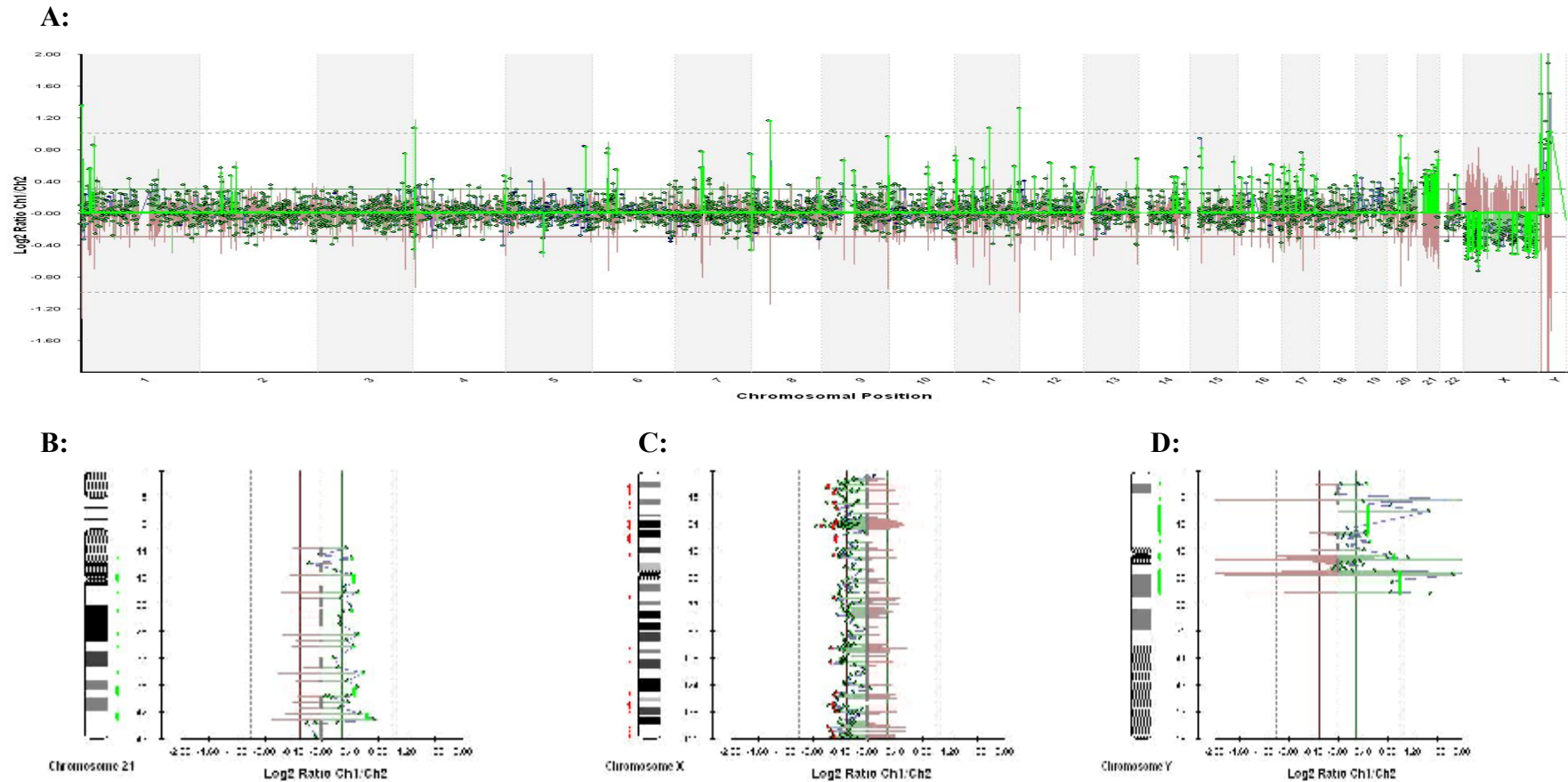
When CoF 8 was amplified with GenomePlex, sex mismatch was detected with all array slides, even though most clones did not reach the log<sub>2</sub> ratio threshold for amplification of the X chromosome. No mosaicism was detected in the placenta sample after FISH. The copy number changes in the X chromosome were based on the location of the majority of spots on the graph which were located towards the amplification region rather than being detected by the software program. Only Y chromosome deletion could be detected with the MDA product and the Spectral Genomic slides.

#### **5.6.4.4 A-CGH analysis of CoF 10**

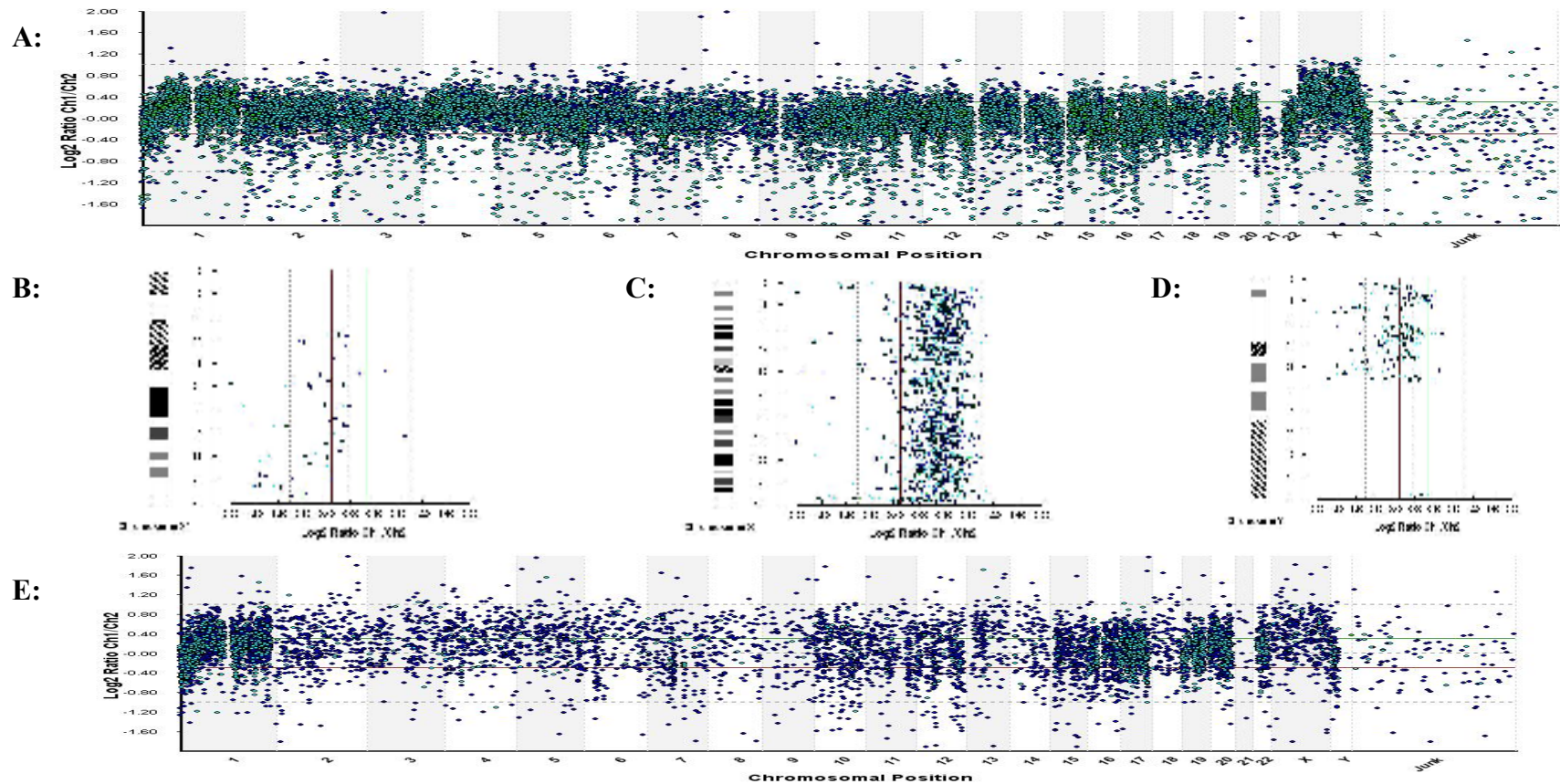
In contrast to the TOP samples, chromosomal abnormalities were expected in the ERPC samples. For CoF 10, the karyotype of the embryo was found to be 47, XY+21 after CVS analysis that was performed clinically. For this reason FISH analysis was not performed on the placenta. Six a-CGH experiments were performed with CoF 10 MDA and GenomePlex products. In the first experiments the MDA sample was hybridised against a normal female control on a Cytochip 1MB slide and a Mermaid 32K array slide. Another MDA sample was produced in which the initial DNA source was 5µl of MDA CoF 10 (re-MDA CoF 10). This MDA sample was hybridised on a Mermaid 32K array slide.

This experiment was done in order to conclude if MDA could reliably be re-amplified as it has been previously suggested by other groups (Alan Thornhill, personal communication). The result was compared with the previous experiment with the 32K Mermaid array. The results are presented in figures 5.9 and 5.10. For the 32K Mermaid arrays, dye-swap was not performed; Cy3 was used for the reference DNA and Cy5 for the test DNA.

From the result obtained from MDA CoF 10 on the Cytochip 1MB slide it can be concluded that overall a-CGH analysis can be performed in DNA extracted from 1ml CoF and be able to detect whole chromosome changes or chromosomal imbalances of at least 50MB, which is the amount of DNA in chromosome 21. It should be noted that the DNA concentration found after DNA extraction of the coelomic fluid was 12ng/ $\mu$ l ( $260/280 = 1.68$ ) which was the smallest amount of DNA from all the samples (table 5.5). From this result an imbalance on chromosome 21 was obtained together with artefacts located all around the genome but preferentially in the chromosome ends. Amplification of the region 1p36 that was detected in this experiment was also abundant in the other experiments discussed in chapter 4 (figure 4.9 (experiment B7) and figure 4.14 (experiment C7)). Analysis of the same sample with the 32K Mermaid arrays did not produce equivalent results but still imbalances on chromosomes 21, X and Y could be identified. Analysis of the re-MDA CoF 10 only included 23.3% of clones and so an outcome could not be concluded. Table 5.9 summarises the data obtained from the analysis of these experiments.



**Figure 5.9:** A-CGH analysis of CoF 10 MDA products with Cytochip 1MB slides. The graph (A) and the ideograms for chromosomes 21 (B), X (C) and Y (D) show the analysis of a-CGH of the sample CoF 10 that was known to be male with trisomy 21. A-CGH analysis with Cytochip 1MB slide can correctly predict the result even though the experiment cannot be trustful for small chromosome changes as a number of clones scattered on the graph would exceed the threshold for amplification/deletion.



**Figure 5.10:** *A-CGH analysis of CoF 10 MDA products with 32K Mermaid slides. Graph A shows the results of a-CGH of CoF 10 MDA product that was applied to a 32K Mermaid slide and the ideograms B, C and D show the detailed result for chromosomes 21, X and Y respectively. The result is much more noisier compared to the Cytochip 1MB experiment but still the majority of the clones of chromosome 21 are detected as deleted. Graph E shows the a-CGH result of an MDA sample that used as DNA source the MDA sample CoF10 in which no reliable outcome can be considered due to the poor hybridisation.*

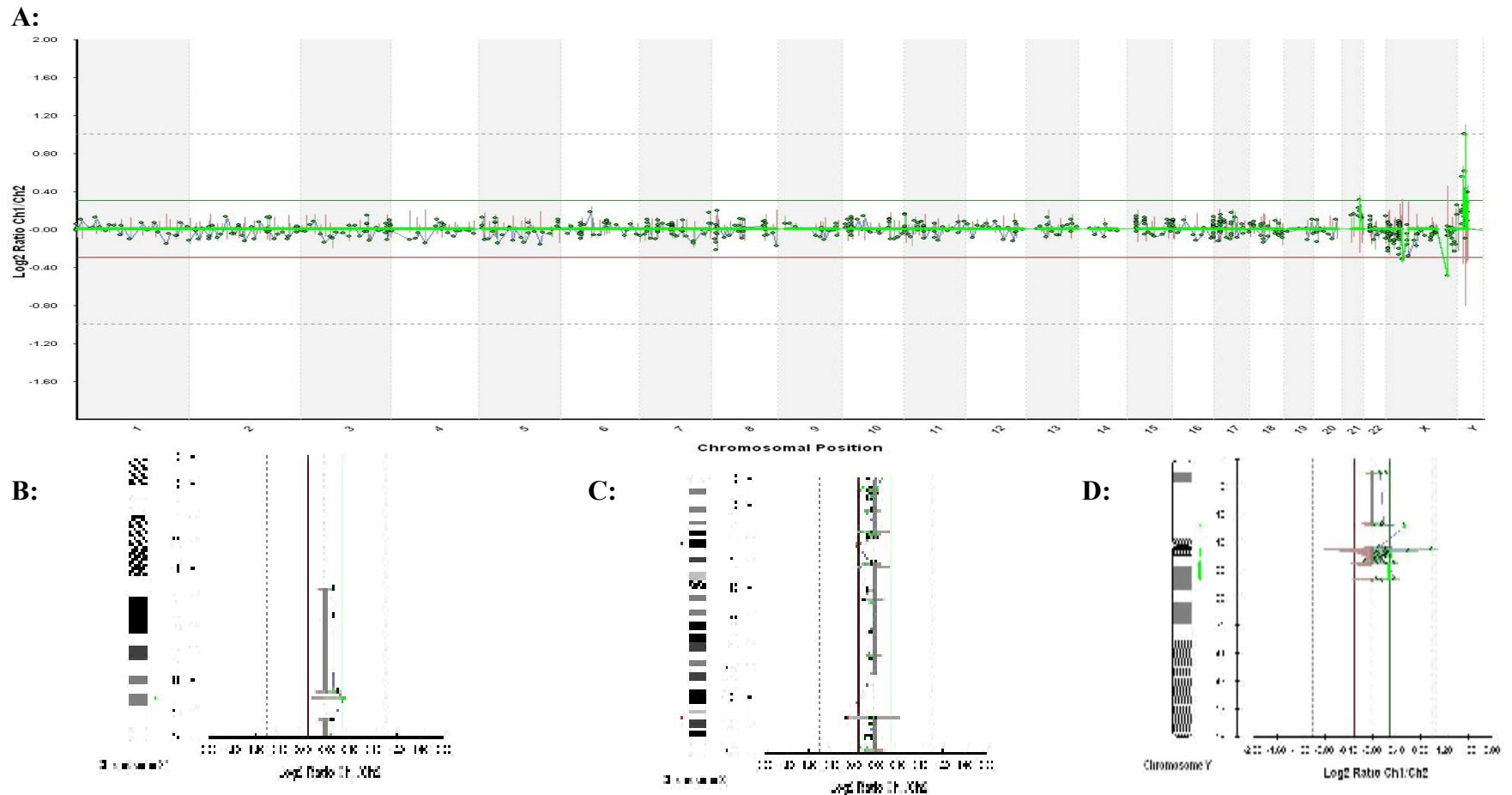


	<b>CoF 10 MDA</b>		<b>CoF 10 re-MDA</b>
	Cytochip 1MB	Mermaid 32K	Mermaid 32K
SD for autosomes	0.174	0.471	0.467
SD for chromosome X	0.137	0.451	0.478
SD for chromosome Y	0.815	0.557	0.386
SD for chromosome 21	0.215	0.828	1.012
Median between replicates	0.05	0.51	0.7
Clones included (%)	99.3	59.9	23.3

**Table 5.9: Summary of results of the a-CGH with CoF 10 MDA products.** This table presents the data analysis from three a-CGH experiments performed with CoF 10 MDA product and CoF re-MDA product. The CoF 10 re-MDA-Mermaid 32K array contained only 23.3% of the clones for analysis so the result was not considered reliable. High SD of the CoF 10 MDA- Mermaid 32K array was also present compared to the CoF 10 MDA-Cytochip 1MB experiment.

Molecular cytogenetic analysis of the GenomePlex CoF 10 was performed with the Cytochip prenatal slide. The result presented in figure 5.11 shows that SD was very low when using GenomePlex and only one spot exceeded the log2 ratio for chromosomal abnormality for chromosome 21. Different analysis settings should be used for detection of trisomy 21 by the software programme. Details of the data obtained are summarised on table 5.10.

As MDA CoF 10 showed the best results with the Cytochip 1MB slide, the next experiment was to produce an MDA sample from which the initial DNA template would be 2.5µl of CoF 10 DNA, rather than 5µl that was used in the previous amplification. That experiment was done in order to conclude if even less CoF DNA would be enough for successful a-CGH analysis. The sample that was produced was hybridised to a Cytochip 1MB slide, exactly the same way as the first experiment (figure 5.8). The analysis of this a-CGH showed a similar pattern with the first experiment but with higher background noise which made the analysis more difficult. However, analysis with the prenatal algorithm provided by BlueGnome showed a clear trisomy 21. The graphs of the result are presented on figure 5.12.



**Figure 5.11:** A-CGH analysis of CoF 10 GenomePlex products with the Cytochip prenatal slides. Graph A shows the results of a-CGH of CoF 10 GenomePlex product that was applied to a Cytochip prenatal slide. Ideograms B, C and D show the detailed results for chromosomes 21, X and Y respectively. Lowering the log<sub>2</sub> threshold ratio for amplification/ deletion would be necessary for the identification of detection of imbalances for chromosomes 21 and X.

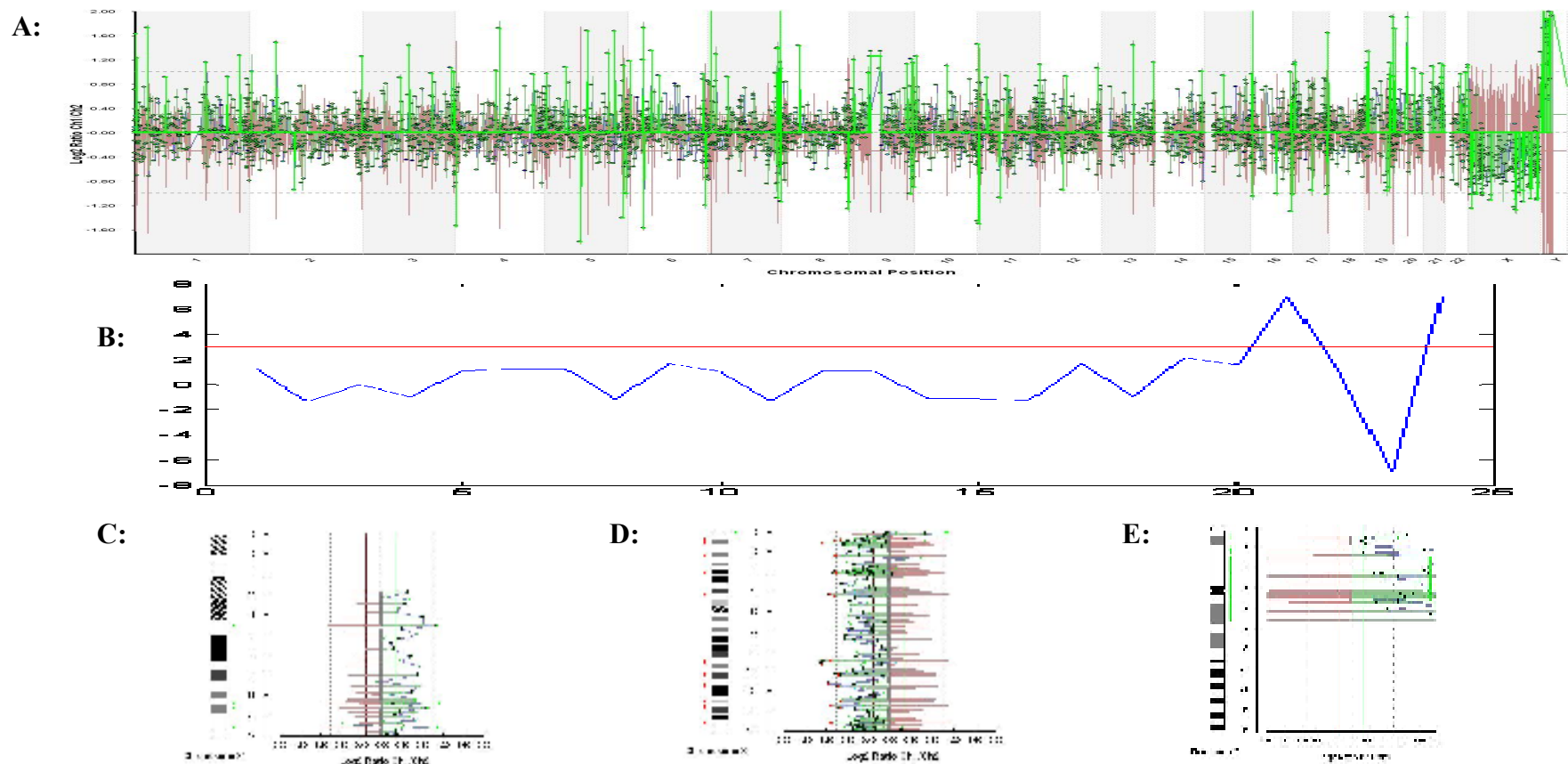


The final experiment that was performed with CoF 10 sample was to test if a-CGH could be successfully performed in minute amounts of DNA from CoF samples, without previously having DNA extraction. MDA was applied directly to 5µl of CoF 10 without previous DNA extraction. MDA protocol 4 (P4) was used with an alkaline lysis. The maximum amount of DNA expected to be present was from 50 cells (because a maximum of 10 cells/µl has been reported for coelomic fluid). The MDA product contained 89ng/µl of DNA. The MDA sample was hybridised against a normal male MDA sample on a Cytochip prenatal slide and the slide was analysed with the BlueFuse Cytochip protocol and with the prenatal algorithm. The result is shown on figure 5.13.

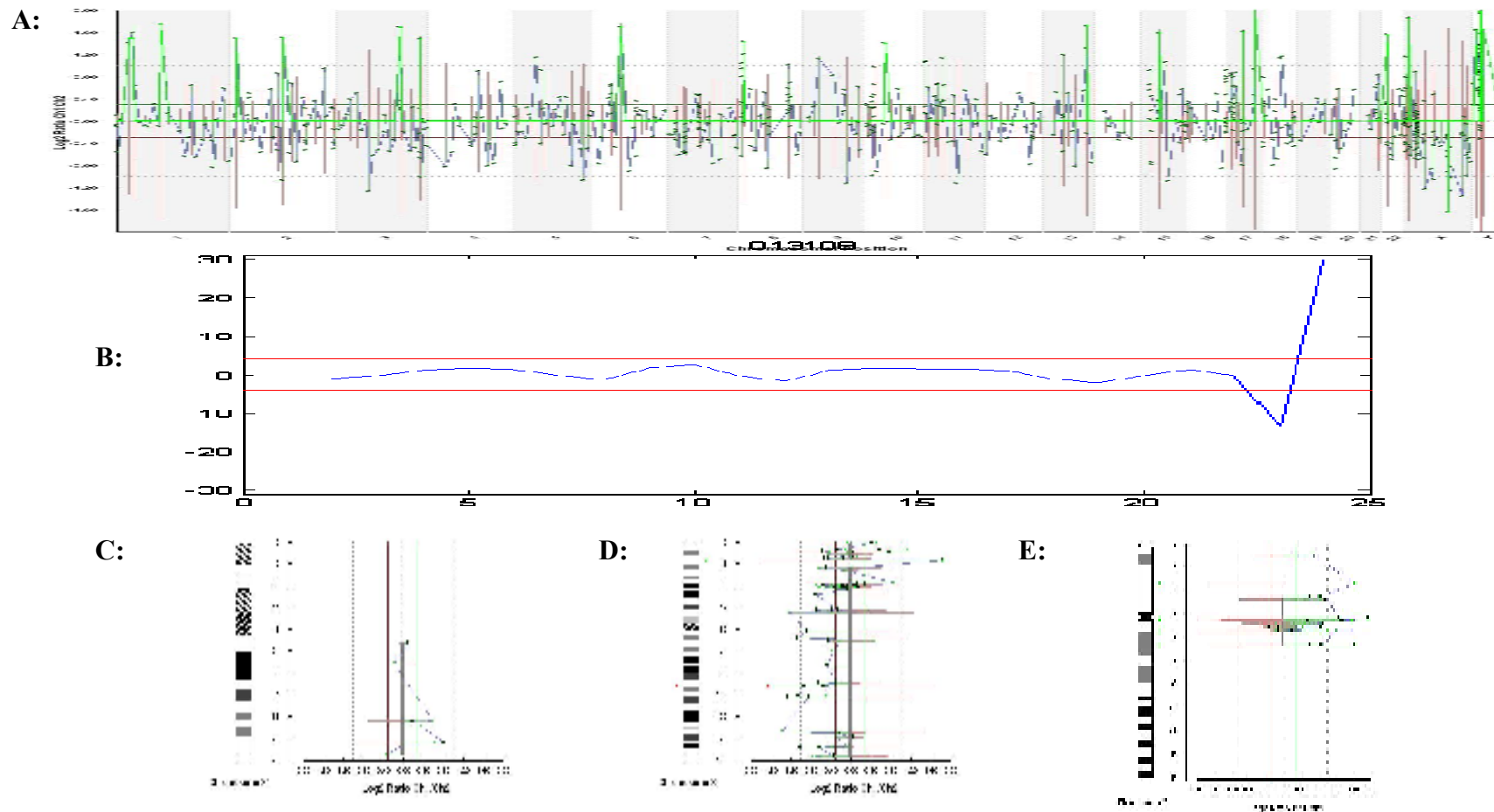
	<b>CoF 10 GenomePlex</b>	<b>CoF 10 half amount MDA</b>	<b>CoF 10 direct MDA</b>
	Cytochip prenatal	Cytochip 1MB	Cytochip prenatal
SD for autosomes	0.071	0.366	0.520
SD for chromosome X	0.098	0.360	0.499
SD for chromosome Y	0.198	1.050	0.698
SD for chromosome 21	0.060	0.319	0.365
Median between replicates	0.03	0.05	0.08
Clones included (%)	99.1	98.56%,	99.26%,

**Table 5.10: Summary of results of the a-CGH with CoF 10 MDA and GenomePlex products.** This table summarises the results of three experiments with CoF 10 sample. The SD of the CoF 10 direct MDA – Cytochip prenatal experiment was the highest among all the experiments with CoF 10 which indicates the low amplification efficiency of MDA when applied directly to CoF.

Unfortunately, the experiment did not give the expected result. The background noise was very high even though a prenatal slide was used. In the PCR for the DM triplex good results were obtained which means that direct application of CoF samples with MDA is feasible for molecular analysis. However, application of this MDA product for analysis by a-CGH was only enough to detect 1:2 changes (X chromosome) and 1:0 changes (Y chromosome) but not 2:3 changes (chromosome 21). Further investigation would be needed for optimisation of direct amplification with MDA of CoF samples.



**Figure 5.12: A-CGH analysis of CoF 10 MDA product from 2.5 $\mu$ l of DNA with the Cytochip 1MB slide.** A: Graph presenting the result of the MDA product from 2.5 $\mu$ l of CoF10 DNA applied on Cytochip 1MB slide. The result has higher SD compared to the experiment where double amount of DNA was used for MDA amplification. Thus, identification with trisomy 21 is more difficult without the use of the prenatal algorithm. B: The same result analysed with the prenatal algorithm showing that trisomy 21 is detected. Ideograms for chromosomes 21, X and Y are presented on figures C, D and E respective.



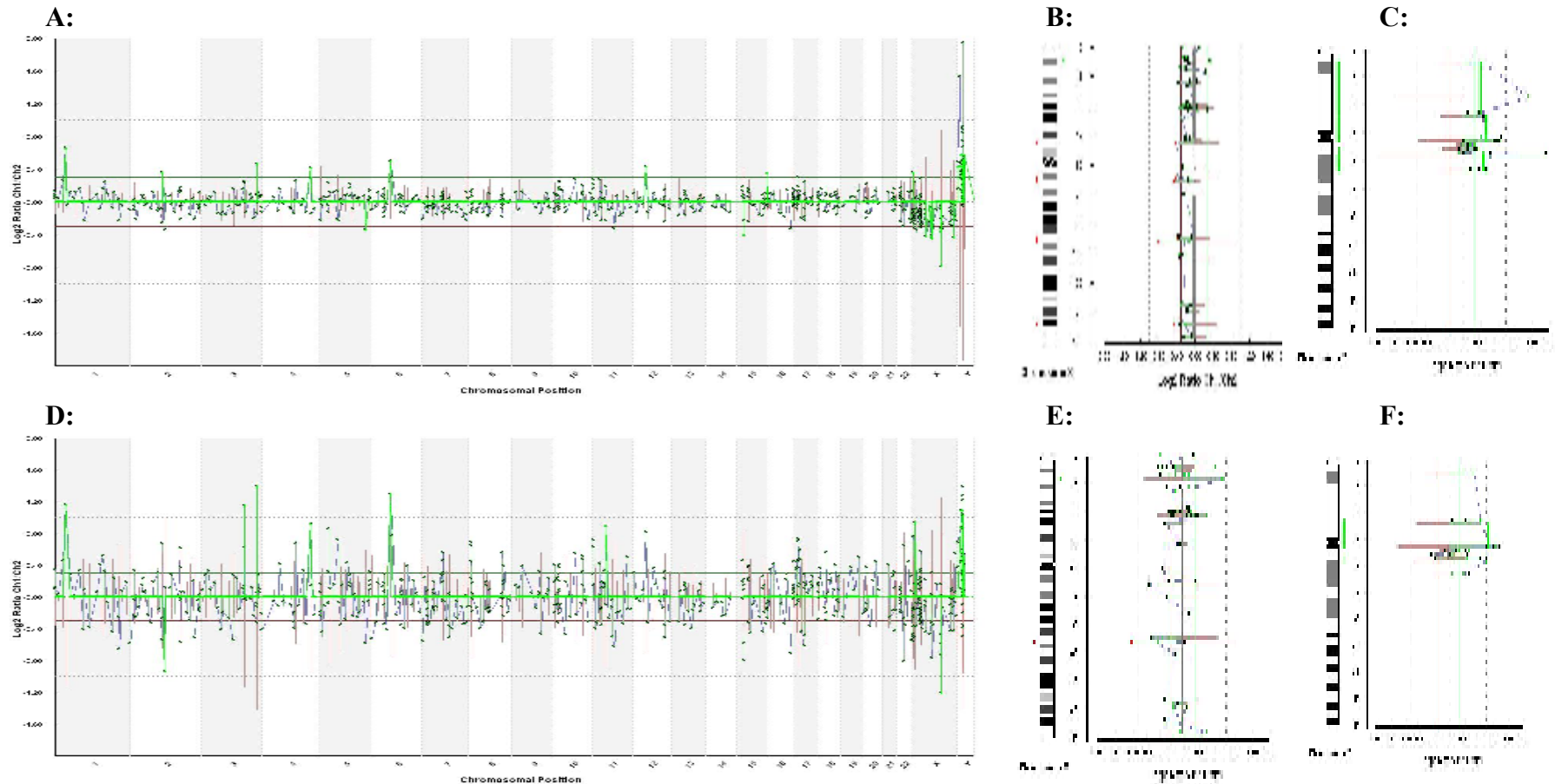
**Figure 5.13:** A-CGH analysis of MDA product derive from direct application on 5 $\mu$ l of CoF10. A: Graph presenting the result of the CoF 10 sample after direct amplification of 5 $\mu$ l with MDA applied on Cytochip 1MB slide. B: The same result analysed with the prenatal algorithm in which only sex mismatch was identified. C, D and E present the ideograms for chromosomes 21, X and Y respectively.

#### 5.6.4.5 A-CGH analysis of CoF 11

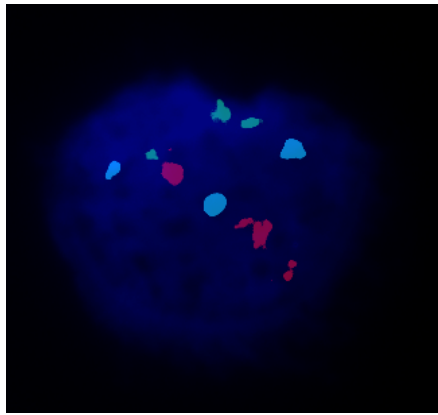
The final CoF sample that was used for molecular cytogenetic analysis was CoF 11. This sample was an ERPC sample from which FISH analysis of the fetal placenta showed chromosomal abnormalities. As two signals were obtained for chromosome X, three for chromosome 18 and one for chromosome Y it was concluded that the sample would have at least two excess chromosomes (one X and one 18). The DNA extracted from CoF 10 was amplified with GenomePlex and MDA and applied on Cytochip prenatal slides. Normal female DNA was used as a reference sample, amplified in a similar way as the test sample. The results from these experiments are shown on figure 5.13. GenomePlex product produced much less background noise compared to the MDA product and showed a much clearer Y chromosome amplification (figure 5.14C and 5.14F, table 5.11). Both results showed a very weak deletion for chromosome X but did not show any amplification for chromosome 18. According to the FISH result, it was expected that the sample should show at least an imbalance for chromosome 18 unless the sample was triploid (69, XXY). In order to confirm this, FISH analysis for chromosomes 13, 15 and 21 was done on the placenta tissue. The results showed that three signals for all the autosomal chromosomes were present which confirmed that the sample was male triploid (figure 5.15).

	<b>CoF 11 GenomePlex</b>	<b>CoF 11 MDA</b>	<b>CoF 11 GenomePlex</b>
	Cytochip prenatal	Cytochip prenatal	32K Mermaid
SD for autosomes	0.124	0.314	0.302
SD for chromosome X	0.154	0.373	0.285
SD for chromosome Y	0.407	0.479	0.493
Median between replicates	0.04	0.12	60.85
Clones included (%)	99.41	91.11%,	0.42

**Table 5.11: Summary of results of the a-CGH with CoF 11 WGA products.** This table presents the data provided by the analysis software from three a-CGH experiments performed with CoF 11. The standard deviation (SD) of the Cytochip prenatal slides was higher in both experiments compared to the equivalent experiments performed in this study.



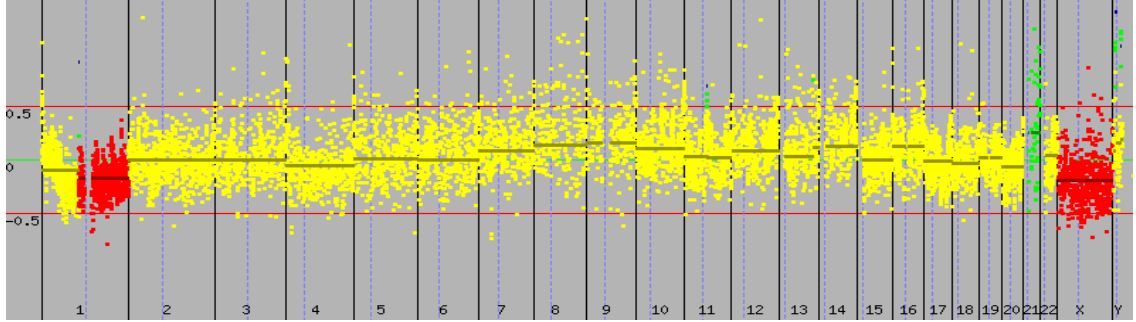
**Figure 5.14:** A-CGH analysis of CoF 11 GenomePlex and MDA products with Cytochip prenatal slides. A-CGH results of the CoF 11 product after GenomePlex (A) and MDA (D). Ideograms B and C show the result for chromosomes X and Y with GenomePlex amplification and E and F the ideograms of chromosomes X and Y after MDA amplification. Both results have higher SD compared to the other Cytochip prenatal experiments but weak sex mismatch is obtained.



**Figure 5.15: FISH analysis of placenta CoF 11 for chromosomes 13, 15 and 21.** Three signals were obtained for each chromosome which implies that the embryo was triploid. Chromosomes 13 (Spectrum green), 15 (Spectrum aqua) and 21 (Spectrum red).

The weak deletion obtained on chromosome X was explained by the way the a-CGH software calculated chromosomal imbalances. The software considers that for the majority of the autosomal chromosomes the ratio between the test and the reference DNA should be 2:2 which is equal to 1. So in a triploid sample where the true chromosomal ratio between test and reference DNA is 3:2 the software will calculate again as equal to 1. This means that for the X chromosome where the ratio between test and reference is 2:2 the software will calculate it as  $<1$ . That is the reason why chromosome X showed a weak deletion in the graph. Although the correct result was not obtained from this sample, it was expected as CGH techniques cannot detect chromosomal balanced abnormalities and triploidies.

The final a-CGH experiment included the application of CoF 11 GenomePlex to a 32K Mermaid array slide in order to compare the result with the Cytochip prenatal slide. The results are shown on figure 5.16 and table 5.11. Unfortunately, from the result obtained a weak deletion was observed in chromosome X and 1q and weak amplification on chromosomes 21 and Y. The high SD of the array was expected as the CoF 11 GenomePlex also produced high SD with the Cytochip prenatal slide, in contrast to all the other CoF GenomePlex samples that were applied to the Cytochip prenatal slide.



**Figure 5.16: A-CGH analysis of CoF 11 GenomePlex product with 32K Mermaid slide.** This graph shows the result obtained from CoF 11 GenomePlex product on a 32K Mermaid array. Regions in red are identified as deleted, regions in green are identified as amplified and regions in yellow are identified as balanced. In contrast the karyotype of the sample from this a-CGH experiment showed deletion of region 1q and X and amplifications of chromosomes 21 and Y.

A summary of all the experiments performed with CoF samples, indicating the WGA method, the a-CGH slides used, the a-CGH result and the fetal karyotype is included in table 5.12.



Sample	WGA method	a-CGH slide	Result from a-CGH	Fetus karyotype	Comparison of results
CoF 4	MDA	Cytochip 1MB	46, XY	Expected 46, XY	Concordant
CoF 4	MDA	Cytochip prenatal	46, XY	Expected 46, XY	Concordant
CoF 4	GenomePlex	Cytochip 1MB	46, XY	Expected 46, XY	Concordant
CoF 4	GenomePlex	Cytochip prenatal	46, XY	Expected 46, XY	Concordant
CoF 7	MDA	Cytochip 1MB	46, XX	Expected 46, XX	Concordant
CoF 7	GenomePlex	Cytochip 1MB	46, XX	Expected 46, XX	Concordant
CoF 7	GenomePlex	Cytochip prenatal	46, XX	Expected 46, XX	Concordant
CoF 8	GenomePlex	Cytochip prenatal	46, XX	Expected 46, XX	Concordant
CoF 8	GenomePlex	Mermaid 32K	46, XX	Expected 46, XX	Concordant
CoF 8	GenomePlex	Spectral Genomics	46, XX	Expected 46, XX	Concordant
CoF 8	MDA	Spectral Genomics	45, X	Expected 46, XX	Disconcordant
CoF 10	MDA	Cytochip 1MB	47, XY +21	47, XY +21	Concordant
CoF 10	MDA	Mermaid 32K	47, XY +21	47, XY +21	Concordant
CoF 10	Re-MDA	Mermaid 32K	inconclusive	47, XY +21	-
CoF 10	GenomePlex	Cytochip prenatal	47, XY +21 (weak amplification)	47, XY +21	Concordant
CoF 10	MDA 2.5µl DNA	Cytochip 1MB	47, XY +21	47, XY +21	Concordant
CoF 10	Direct CoF MDA	Cytochip prenatal	46, XY	47, XY +21	Disconcordant
CoF 11	GenomePlex	Cytochip prenatal	46, XY (mosaic)	69, XXY	Disconcordant
CoF 11	MDA	Cytochip prenatal	46, XY (mosaic)	69, XXY	Disconcordant
CoF 11	GenomePlex	Mermaid 32K	47, XX -1p +21	69, XXY	Disconcordant

**Table 5.12: Summary of a-CGH for this chapter.** This table summarises all the a-CGH results performed for this study and compares them with the expected karyotypes of the fetuses. For all the TOP samples no chromosomal abnormalities were expected and the a-CGH results detected the expected sex of the fetus in most of the cases. The triploid sample was not detected by any type of array or WGA method.



## 5.7 Discussion

This study focused on chromosome analysis of CoF samples using a-CGH. The advantage of achieving reliable CoF diagnosis is correlated with the advantages of early prenatal diagnosis from the 5<sup>th</sup> week of gestation. Specific problems arise with CoF collection and analysis, some of which I tried to be addressed in this project. The crucial problem of defining the actual fetal loss rate following coelocentesis could not be addressed here as it is a medical aspect.

Maternal contamination is an issue that raises questions of the origin of the DNA from the CoF samples. Contamination has to do with the content of the CoF but also with the technique by which the samples are aspirated. A solution for avoiding contamination in respect to coelocentesis would be to discard more than 0.2 ml of CoF fluid. In this project, the contamination problem was avoided by only choosing samples that were validated for their purity with the use of PCR. A total of six out of the 13 samples were defined as pure. For the rest of the samples I could not determine whether they were pure or contaminated but because all three alleles matched the maternal alleles, the chance of contamination existed and so they were excluded from analysis. All of these samples were light or dark yellow in colour. CoF sample normal colour is clear transparent and when the colour appears to be light or dark yellow indicates that the sample contaminated by blood. Most of the other studies performed regarding PCR and FISH analysis have found small or high contamination of CoF samples (Jouannic et al., 2006 and 2008; Jauniaux et al., 2003) even in samples where the CoF colour is clear.

The technical difficulty in this study was the quantity and quality of CoF DNA obtained and the application on a-CGH. So far, studies have reported PCR (Jauniaux et al., 2003) and FISH analysis (Chatzimeletiou et al., 2004 and 2005) but this was the first application of a-CGH analysis on CoF samples. The crucial point of this study was the different WGA applications and the comparison with different array slides and

resolutions. The amount of DNA available for diagnosis, the WGA techniques and the different array slides and resolution analysis are discussed in the following pages.

### **5.7.1 Concentration CoF DNA and minimum amount for WGA**

It has been found that CoF samples have a wide variability of cells density, ranging from 0 to 10,600 cells/ml but also contain cell free DNA (cfDNA) (Jouannic et al., 2008). Since each cell contains 6pg DNA the maximum amount of DNA from CoF cells is around 60pg/ $\mu$ l. Real-time PCR analysis showed a large variation of the final amount of DNA present after DNA extraction which can also be explained by the large variation of the cfDNA. In this study, measuring DNA by NanoDrop was not considered to be an accurate measurement as the minimum detection limit proposed by the Instrument's manual is 2.5ng/ $\mu$ l. Nevertheless this was not of such great importance for our study as WGA was applied to the samples. The DNA extraction protocol that was performed for this project focused on extracting DNA from cells and not the cfDNA as DNA extraction was applied after discarding the supernatant following centrifugation. CfDNA is abundant in the supernatant and has been found to be degenerate DNA of very small molecular weight (Larrabee et al., 2004) and it is most likely to be of fetal origin (Makrydimas et al., 2008). The MDA sample that was applied directly to CoF without DNA extraction (CoF 10, section 5.6.4.4) probably amplified cfDNA together with DNA from a few cells.

Although up to 4ml were collected for analysis, only 1ml was used for DNA extraction according to suggestions by other groups that minimising the amount of CoF could reduce the fetal loss rate (Jouannic et al., 2006). As DNA can vary a lot following DNA extraction it was important to set a minimum amount that could be used for WGA and consequently for a-CGH analysis. For this reason in one sample where trisomy 21 was detected, WGA was repeated on 2.5 $\mu$ l of CoF DNA template in contrast to the 5 $\mu$ l of DNA used in all other samples. Minimizing the amount of DNA used, direct amplification of CoF was also performed with CoF 10, even though the results were not successful. An interesting experiment would be to isolate single or a small number of

CoF cells under the microscope, as in single cell isolation, and then apply this to WGA for a-CGH analysis. This was not performed as expertise was needed in order to identify and distinguish CoF cells from the presence of other cells (eg. maternal cells from blood contamination). The knowledge acquired from the single cell a-CGH chapter (chapter 4) would have been very beneficial for successful analysis of this kind of experiment.

### **5.7.2 Different WGA methods for CoF DNA amplification**

Two methods of WGA were used, MDA and GenomePlex. Both were used in the single cell a-CGH chapter and managed to correctly amplify DNA from a single cell, at least for the sex mismatch discrimination. As the DNA quantity obtained from these samples was greater compared to a single cell, better results were expected. Most studies conclude that MDA is superior to GenomePlex for genome-wide studies when the input DNA is from good quality (Park et al., 2005; Barker et al., 2004). However, according to the literature, GenomePlex has advantages over MDA for samples of poor DNA quality (Leanza et al., 2007). In a study by Mead et al. (2008) GenomePlex was compared with MDA for the amplification of degraded DNA with high-throughput SNP arrays and concluded that overall GenomePlex presented better results. The advantage of GenomePlex has to do with the ability to amplify DNA of poor quality, such as formalin fixed ethanol preserved (FFEP) samples, better than MDA. A second advantage is that GenomePlex products do not produce non-specific DNA, in contrast to MDA. Experimental proof for this is the presence of DNA after amplification of DNA-free samples with MDA (Hughes et al., 2005). This is an explanation of the high background noise with MDA samples in the a-CGH experiments. Generally in the results from this chapter, GenomePlex products had a much lower SD, but in certain samples (CoF 7 and CoF 8) the chromosomally unbalanced regions of test and reference DNA did not always exceed the threshold for amplification or deletion.

As other groups suggest, the DNA quality obtained after MDA is crucial and can predict the quality of an a-CGH result (Buffart et al., 2007). According to a study by Buffart (2007) high DNA yield after MDA amplification ( $>10\mu\text{g}$ ) is an accurate predictor for

good a-CGH results. In the results from this chapter, only MDA amplification of 20ng of gDNA resulted in overall DNA close to 10mg (control male =9.24mg DNA, control female = 9.16mg DNA). The results obtained from hybridisation of the MDA male and female control products on the Cytochip 1MB slide are presented on figure 4.7, had the lowest SD (SD = 0.133) and presented a good sex mismatch difference. For all the other MDA samples that were used for a-CGH experiments the final DNA yield varied from 1.66mg to 3.8mg.

An interesting modification of the MDA technique has been proposed to overcome the problems encountered with degraded and damaged DNA. The technique developed is named restriction and circulisat-ion- aided rolling circle amplification (RCA-RCA) and uses enzymes to cut and ligate the DNA into circular DNA template prior to MDA in order to avoid duplex ends. This technique was first described by Wang et al. (2004) and results reported so far are very promising (Wang et al., 2004). RCA-RCA should be ideal for applying to DNA from CoF samples. This technique would be even more helpful for the direct amplification of CoF samples without DNA extraction as the majority of the DNA present is expected to be cfDNA (like CoF 10). Unfortunately, this technique was not used in this project due to high cost and minimum number of a-CGH experiments but it should be used in future research.

### **5.7.3 Molecular cytogenetic analysis of CoF by a-CGH**

Genetic diagnosis in prenatal samples was first reported in 1956 (Fuchs and Riis, 1956). The development of different techniques for more secure collection of samples and different genetic tests for more accurate and fast diagnosis has not stopped since then. Recently, a-CGH was introduced in the analysis of prenatal samples (Le Caignec et al., 2005; Rickman et al., 2005; Larrabee et al., 2004; Schaefer et al., 2004).

The evolution of a-CGH towards high resolution analysis raised critical issues for prenatal diagnosis use. Copy number variants (CNVs) exist in the genome and they are not always connected to clinical malformations (Perry et al., 2008). Detection of a not

previously characterised chromosomal imbalance would raise concerns regarding the fate of the fetus (Vermeesch et al., 2005). For this reason, a-CGH slides with target probes located on or close to known chromosomal abnormalities have been developed with average resolution 5-10MB. In this project, these types of slides were used but also 1MB and tiling path array slides were applied to WGA products from CoF samples. Nevertheless, all the slides were used for detection of large chromosome changes. As in the single cell a-CGH project, Cytochip prenatal and 1 MB slides provided the most accurate and reliable results.

Guidelines for molecular karyotyping for prenatal diagnosis have been published (Vermeesch et al., 2007). According to these guidelines several experiments involving self-self hybridisations, replicate experiments, sex mismatch and chromosome add-in experiments have to be performed in order to estimate the sensitivity and the specificity levels of specific a-CGH slides in a laboratory. In this project, only sex mismatch was performed due to the high cost of experiments and because no clinical application would be performed. However, this study focused only on the application of CoF WGA products to a-CGH analysis for large/whole chromosome changes and not on the analysis at the theoretical resolution of each slide. Although it was not a blind study, a trisomy 21 was detected by the software analysis which can set the experimental resolution to 50MB. Due to the many artefacts present, a large number of false positives were predicted by the software analysis in the 1MB and the tiling path arrays but these would not have been detected if the prenatal algorithm was applied to all samples (as it was performed with CoF 10 samples, figure 5.10). Only the combination of GenomePlex products and Cytochip prenatal slides produced results where there were no or very few false positive results. However, in these experiments the log2ratio for amplification/deletion had to be decreased for the detection of imbalance by the software.

The detection of a region to be identified as deleted or amplified was based on the majority of the clones located on a specific chromosome being above or below the threshold level. Imbalances may be identified if the post analysis measurements were changed but Cytochip analysis for the Bluefuse Cytochip analysis would not allow any

modification in the settings. The software from BlueFuse could not always identify deletions and amplifications and erroneously identified normal regions as deletions/amplifications due to artefacts and high SD, especially in the MDA samples. However, in the CoF 10 sample (figure 5.12) analysis with the prenatal algorithm demonstrated that the trisomy can be detected if a different algorithm is used for the analysis.

An important issue regarding the success of an a-CGH experiment is the total SD. The only control experiment that was performed with MDA of good quality DNA with Cytochip 1MB slides was presented in chapter 4 where the SD was 0.133. This should be the minimum SD expected by Cytochip 1MB slides and MDA products. Unfortunately, no equivalent experiment was performed with GenomePlex or with different array slides. In order to minimise the SD, the reference samples were treated in a similar way as the test samples as has been suggested by various groups (Knijnenburg et al., 2007; Arriola et al., 2007). Another important issue with the success of an a-CGH experiment is the final clone inclusion for the analysis. In all experiments, apart from the ones performed with the 32K Mermaid arrays, the total clone inclusion exceeded 90%, which is the limit suggested by the guidelines. The 32K Mermaid arrays have been developed for research purposes and so clone inclusion >70% was considered to be acceptable.

#### **5.7.4 Conclusion**

In conclusion, reliable aneuploidy screening of all chromosomes with a-CGH can be achieved. The prenatal algorithm could provide reliable results for whole chromosome changes avoiding high SD produced by WGA. More experiments would be necessary for determining the resolution that analysis can be performed.

## **Chapter 6**

### **Conclusions**

#### **6.1 Diagnosis with single cells and small numbers of cells**

The main problem with PGD arises from the minimum amount of DNA present for diagnosis and currently the only technique that helps to overcome this problem is WGA. The future of PGD is to make WGA a universal step prior to any genetic test so that PGD can be performed using direct and indirect mutation markers, PGH, a-CGH or by combining PGS and PGD for monogenic diseases. WGA could also be useful for the study of chromosome mechanisms (compaction stages, meiosis, crossing-over) during early development and the investigation of more complex genetic mechanisms, such as imprinting which has been found to play a crucial role in assisted reproductive technology (ART) (Huntriss et al., 2008).

Similar to single cell diagnosis, coelocentesis is hampered due to low DNA concentrations present in the coelomic fluid collected. If this problem could be solved, the advantages of early diagnosis by coelocentesis would be safer termination in cases of affected fetuses and fetal treatment before the fetus becomes immunologically intolerant. The way to overcome the low amount of DNA is WGA.

#### **6.2 WGA for single cells and small numbers of cells**

When applying WGA to a single cell, optimisation of each step would be essential for robust amplification. In chapter 3 it was shown that even a small modification of the lysis protocol can alter the WGA efficiency (exclusion of DTT, L1 method). It was also shown that different cell types and lysis methods may respond differently to WGA. This demonstrates the importance of optimisation of a technique prior to any clinical or research application. In studies where different cell types are used (cancer studies,

forensic studies, archaeological studies) testing of different lysis methods should be applied. In chapter 3, the L1 lysis method gave the best results and buccal cells were shown to contain poor quality DNA.

There are several WGA methods developed but all of them are based on either PCR or MDA. MDA and GenomePlex were used in this project because they have been reported to be the more promising for single cell amplification (Fiegler et al., 2007; Le Caignec et al., 2006). Other techniques used to amplify single cells are DOP-PCR, PEP, RCA-RCA and LA-adaptor PCR. For all these techniques, apart from GenomePlex, there are no protocols specialised for single cells provided by the manufacturers and the minimum requirement of DNA input is 1ng.

For molecular analysis at the single cell level only MDA can be used as it is the only method that produces long DNA strands and can be reliable for molecular analysis. GenomePlex was very recently reported as the WGA method for PGD with PCR and direct sequencing (Chen et al., 2008). The authors reported a total of 6-12% TAF and 6-19% ADO of the 16 GenomePlex products from single blastomeres for five PCR reactions (one for  $\beta$ -thalassaemia and four for HLA haplotyping). One ongoing pregnancy was achieved from two cycles of embryo transfer. Nevertheless, I do not consider this technique reliable due to the fragmentation step that is needed for the amplification of the genome. MDA has already been applied in clinical PGD and several reports have referred to the benefits of the amount of DNA obtained (Renwick et al., 2006; Burlet et al., 2006). However the disadvantage of the technique is that the results obtained are worse compared to optimised single cell protocols (chapter 3). Apart from this, the results reported from different groups vary and each group reports small modifications regarding the lysis buffers and amplification time. Very few papers have been published with extensive analysis of single cell MDA molecular analysis (Glentis et al., 2009; Spits et al., 2006; Renwick et al., 2006). In contrast, clinical PGD with MDA has been reported by several groups and in some cases very few work-up experiments have been performed. I conclude that the use and optimisation of MDA for PGD



molecular analysis should be focused on PGH or multiple testing, such as the combination of direct and indirect analysis.

For single cell a-CGH various WGA techniques can be used. Due to the nature of a-CGH, different standards of DNA quality compared to PCR are necessary for robust results. For example, single nucleotide miss-incorporations and production of short DNA molecules would not be considered as important as even representation of the genome and non-specific DNA formation. In this study, GenomePlex seemed to provide better results compared to MDA on a-CGH but the disadvantage of this technique was the low amplification efficiency (70%, 7/10) obtained in the results in chapter 4. More experiments will have to be done to measure amplification efficiency of GenomePlex in single cells as this would be a crucial factor for PGD. Comparing the two lysis methods for GenomePlex (L1 and L3) was not possible due to the small amount of experiments that were performed. BlueGnome is currently developing single cell a-CGH protocols in collaboration with UCL Centre for PGD. The single cell WGA protocol consists of a modification of GenomePlex and uses a different polymerase for the amplification of the DNA.

Recently, LA-adaptor PCR was used for single cell a-CGH and the authors claimed to detect imbalances as small as 5MB (Fuhrmann et al., 2008). Nevertheless, the small size imbalances detected were more than single copy (six to nine copies) and the success of this project was mainly due to the optimisation of the array platform. The first clinical PGS with a-CGH was published with the use of two cells instead of one (Hellani et al., 2008).

Most of the WGA kits provided from companies are expected to robustly amplify the DNA of such quantities as seen in CoF. Both methods used managed to show reliable data for whole chromosome changes but GenomePlex provided much clearer profiles with none to very few positive controls and also managed to work on different slides.

### **6.3 A-CGH platforms and analysis**

In this study, only BAC array platforms were used for chromosomal analysis of single cells and CoF. Three different resolution arrays were used (10MB, 1MB and tiling path). Nevertheless, the diagnostic resolution did not increase with the higher resolution slides as the SD increased as well. It seems that with WGA application on such small quantities of DNA it is not possible to achieve the theoretical resolution of the arrays.

For single cell a-CGH only whole chromosome changes could be detected and more work is necessary for reliable results. A trisomy 19 was detected (experiment D4) and was also confirmed by PCR. However, a trisomy 5 that was clearly identified in experiment D2 was not confirmed by PCR. Finally, a trisomy 1 was not detected by the prenatal algorithm even though the majority of the clones had a positive value of  $\log_2 \text{Ch1/Ch2}$  ratio. For the CoF samples, better results were obtained with much less false positive results compared to single cells. According to the results obtained from chapter 5, a-CGH with CoF samples should be considered accurate for whole/large chromosome changes.

An important issue however, has to do with the desired resolution obtained by a-CGH for PGS and prenatal diagnosis. Different slides that offer CGH analysis are oligonucleotide and SNP arrays. The theoretical resolution that can be achieved is very high. Oligonucleotide arrays use small DNA molecules (25-60mer) and each slide may contain hundreds of thousands of target regions that allow much higher resolution than BAC arrays. The background noise of oligonucleotide arrays is much lower compared to BAC arrays. Nevertheless, analysis at 50 -100 kb resolution may allow detection of CNVs but it is not known if they correlate with a genetic defect. Different studies have shown that benign CNVs may range from 24 to 824 (Korbel et al., 2007; Wong et al., 2008; Kidd et al., 2008) and it is likely that “every fetus will be identified as abnormal” if it is analysed by high resolution a-CGH (Shuster, 2007). My conclusion is that low resolution arrays (5-10MB) or low resolution analysis (such as the prenatal algorithm) is more likely to detect the majority of the known genetic malformations and do not produce difficult interpretation or counseling. From a recent clinical application of low resolution a-CGH on prenatal samples it was found that only 1% (3/300) of the diagnosis had a CNV of

unknown clinical significance (Veyver et al., 2009). For PGS, only large/whole chromosome changes should be considered as the main objective of PGS is to increase the pregnancy rate.

SNP arrays are oligonucleotide arrays, but instead of following the CGH procedure, they work by separate hybridisations of the test and the reference DNA and the results are analysed by comparing the embryo and parental SNPs and by measuring the fluorescence intensities of test and reference DNA (Wells et al., 2008). In this way the haplotype of the sample is revealed and a DNA fingerprint is produced. Two recent studies with SNP arrays and single cells (blastomeres, lymphocytes and known cell lines) were published and the results were promising for future application PGS (Scott et al., 2008; Kearns et al., 2008). The potential of SNP arrays for PGS is that chromosomal abnormalities (including uniparental disomy) and single gene disorders can be detected in a single experiment. However, ethical concerns arise with this approach. Genotyping of the embryo will provide a huge amount of information as well as detecting chromosomal abnormalities and single gene defects; these include predisposition to late onset genetic diseases, such as diabetes and cancer or non-disease related characteristics, such as height and skin colour.

In single cell a-CGH, the highest resolution reported was with BAC arrays. Fiegler et al. (2007) reported a resolution of 10MB for single copy changes and Fuhrmann et al. (2008) reported a maximum resolution of 5MB for multi-copy changes. Such high analysis could be very useful for research purposes. In the first clinical PGS a-CGH report an oligonucleotide array platform was used instead of a BAC array but the analysis concentrated on whole chromosome changes rather than high resolution analysis (Hellani et al., 2008). The initial work-up as a-CGH experiments were performed on 20 embryos that had been previously diagnosed with only one chromosomal abnormality by FISH. Five/twenty (25%) results were detected as normal (false negative). Surprisingly the authors concluded that this was due to mosaicism and not due to misdiagnosis although no further investigation was performed. In the clinical application, a total of 41 embryos were assessed with a-CGH from eight PGS cycles and a pregnancy was achieved in 5/6

cycles which had an embryo transfer. Very recently, the CARE fertility clinic in Nottingham reported the first clinical application of polar body a-CGH analysis (in collaboration with BlueGnome) for whole chromosome changes with 1/4 cases resulting in pregnancy. Finally, a prospective “non-selection” PGD trial was performed with the use of a-CGH. In this study, all embryos were assessed by a-CGH but the embryo selection was not influenced by the a-CGH result. It was found that for all the embryos in which a-CGH predicted a chromosomal abnormality either failed to implant or miscarried (100% success) whereas in 42.9% cases where a-CGH did not show any chromosomal abnormality, an ongoing pregnancy occurred (Scott et al., 2008).

The interpretation of the result is also very important. For diagnostic purposes a chromosomal abnormality should always be scored by the analysis software. The result of an experiment depends on certain criteria that are assigned prior to analysis, such as the normalisation protocol and the algorithm used. In WGA products from single cells where the SD is very high, the majority of the clones exceeded the log<sub>2</sub> ratio for amplification/deletion. Analysis with the BlueFuse Cytochip method was shown to be defective for these samples and only analysis with the prenatal algorithm could be considered reliable. Analysis methods, such as the prenatal analysis, estimate the average log<sub>2</sub> ch1/ch2 ratio for a number of consecutive clones at a chromosome in order to estimate chromosome imbalances but consequently the analysis resolution will drop. For the CoF samples, detection of chromosomal imbalances without the use of the prenatal algorithm could be scored when GenomePlex products were applied to the Cytochip prenatal slides.

At the 2008 International Society for Prenatal Diagnosis (ISPD) conference (Vancouver) a-CGH, multiplex ligation-dependent probe amplification (MLPA) and quantitative fluorescent PCR (QF-PCR) were compared for accurate prenatal diagnosis. Results from various groups were presented indicating the advantages and the disadvantages of each technique. The difference between these techniques was that MLPA and QF-PCR provide less information compared to a karyotype whereas a-CGH can provide much more information (with the exception of balanced translocations and triploidies) that require

difficult counselling and extensive follow-up studies (Ogilvie et al., 2009). Nevertheless, karyotyping remains the gold-standard for prenatal diagnosis.

#### **6.4 Future experiments**

WGA by MDA may be used for PGD. Further experiments would include the optimisation of a number of PCR markers linked to a genetic condition using singleplex reactions on single cell MDA products that can be applied to every family according to the markers for which the parents are informative. Although PGH has already been applied it uses a large number of markers that have not been previously optimised for MDA and there is a chance of misdiagnosis due to double recombination events and high ADO (Renwick et al., 2007; Kakourou et al., 2007). However, MDA products could be used for haplotyping with fewer markers which have previously been optimised and are closer to the targeted mutation. No buccal cells and proteinase K lysis should be used with MDA. MDA could also be tested in cases where optimised protocols for PGD exhibit high ADO rates.

The single cell a-CGH study is already evolving. The array experiments in this project were performed at the North East Regional Cytogenetic laboratory but now in UCL Centre for PGD has organised the necessary equipment and materials (apart from the scanner) in order to perform future array experiments on site. Positive control experiments have successfully been performed on site and the slides were scanned at the TRL. Currently, UCL Centre of PGD and BlueGnome are collaborating for further optimisation of a-CGH. A blind study is being performed with single cells isolated from epithelial cell lines with stable chromosomal abnormalities and amplified by a modified protocol based on GenomePlex. BlueGnome developed new BAC-array slides (24sure arrays) that are being used for this project (non-dye swap experiments). The results so far are promising and reproducible for detection of whole chromosome changes (a 47, XX+10 was successfully detected). However, analysis from a complex karyotype (53,X,add(x)(pter), +der(1),+,add(6)(pter), +add(6)(pter), +12, +add(17)(p21), +21, +21,add(22)(p11.2)) failed to detect the majority of the abnormalities and the results were

inconsistent. Due to the way the software does the analyses, it is necessary for the majority of the clones to be chromosomally balanced with the reference DNA. In blastomeres however, chaotic embryos exhibit many chromosomal abnormalities which could affect the analysis of the experiment. More sophisticated analysis algorithms should be developed for complex karyotypes at the single cell level of a-CGH analysis. What is encouraging is that for all the a-CGH experiments with the complex karyotype, the software predicted that the single cell was not normal and so in a clinical case it would be excluded for transfer. The future experiments, which are being performed by another PhD student (Thalia Mamas), will focus on further optimisation of WGA techniques and application of different array platforms and labelling methods.

For future research regarding CoF, the goal would be to concentrate on ERPC samples in order to confirm more chromosomal abnormalities with a-CGH. With the knowledge of single cell isolation, a-CGH analysis could also be performed on an isolated small number of cells, which could ensure the absence of contamination of the samples. Further investigation of the nature of cell free DNA in the samples could also be performed with a-CGH and WGA.

In conclusion, microarrays are a powerful tool of genetics that can provide much information and detail even from a single cell. Together with WGA, new research and clinical horizons have arisen regarding single cell analysis. Whatever methods are used for PGD, ethical dilemmas will occur as PGD can be used for more ethically sensitive situations. It is up to societies and scientists to decide the ethical limitations for PGD.

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## **Appendix A**

### **A.1 Suppliers of chemicals**

The chemicals for this project were obtained from Roche, Sigma, VWR and Gibco BRL unless stated differently.

### **A.2 Solutions for DNA extraction from blood**

The reagents used for the DNA extraction were buffers TKM1 (10 mM Tris-HCl, pH 7.6; 10 mM KCl, 10 mM MgCl<sub>2</sub> and 2 mM EDTA) and TKM2 (TKM2 (10 mM Tris pH 7.6, 10mM KCl, 10 mM MgCl<sub>2</sub>, 0.4 mM NaCl, and 2 mM EDTA), Igepal (Sigma, UK), 10% sodium dodecyl sulphate (SDS; Sigma, UK), 6M NaCl and 1% TE buffer.

### **A.3 Solutions for DNA extraction from coelomic fluid**

The Puregene genomic DNA purification kit (Gentra Systems, USA) was used for coelomic fluid DNA extraction. The kit included a Cell lysis solution, RNase A solution, Protein precipitation solution, Glycogen solution (20 mg/ml) and DNA hydration solution. Proteinase K (20mg/ml) and isopropanol was provided from Sigma.

### **A.4 Materials and solutions for single cell isolation and lysis**

The materials for single cell isolation used were plastic Petri dishes (Sterilin, UK), dissecting microscope, hand pipettes that are controlled through aspiratory micromanipulation and ABI9700 thermocycler. The reagents used for alkaline lysis were dissociation buffer (DB; 140mM NaCl, 0.2mM KCl, 0.04mM NaH<sub>2</sub>PO<sub>4</sub> X 2H<sub>2</sub>O, 5.5mM glucose, 1.2mM NaHCO<sub>3</sub>, 0.02mM EDTA, and 0.01% (w/v) phenol red) (Sigma, UK), bovine serum albumin (BSA, Sigma, Dorset, UK), KOH solution and Dithiothreitol (DTT, Sigma, UK) and for PK lysis phosphate buffer saline (PBS, Sigma, UK), polyvinyl alcohol (PVA, Sigma, UK), Proteinase K and SDS (Sigma, UK).

### **A.5 Materials and solutions for WGA by MDA and GenomePlex**

WGA both for MDA and for the GenomePlex was accomplished with the use of kits from companies. All the WGA reactions were carried out in an ABI9700 thermocycler (Applied Biosystems, UK).

For the MDA reaction, the Repli-g midi kit (Qiagen, UK) was used which contained the Repli-g midi DNA polymerase, the Repli-g midi reaction buffer, the reconstituted DLB buffer, the stop solution and the DTT 1M solution. Nuclease free water was from Promega. Denaturation solution (D1) was 15% reconstituted DLB buffer, denaturation solution D2 consisted of 5µl of 1M DTT and 55µl of reconstituted buffer DLB and N1 solution was 10% of Stop solution.

For the GenomePlex amplification of gDNA the complete WGA kit (WGA2; Sigma, UK) was used which contained the WGA DNA polymerase, the 10X fragmentation buffer, the 1X library preparation buffer, the library stabilization solution, the library preparation enzyme, the 10X amplification master mix and the nuclease-free water. For single cells the Genomeplex single cell WGA kit (WGA4; Sigma, UK) was used which contained the same reagents as the WGA2 apart from the 1X single cell library preparation buffer (instead of 1X library preparation buffer), the 10X single cell lysis and fragmentation buffer (instead of 10X fragmentation buffer) and the Proteinase K solution (not included in the WGA2 kit).

### **A.6 Materials and solutions for PCR**

The primers required for PCR reactions were obtained from Eurogentec Ltd UK. Hifi polymerase (Roche Diagnostics Ltd, UK), 10X Hifi fidelity buffer II (Roche Diagnostics Ltd, UK), dNTPs (Promega, USA), glycerol (Sigma, UK) and nuclease free H<sub>2</sub>O (Promega, USA) were used for the PCR reaction. All PCR Master mixes were prepared in a DNA free room with positive pressure inside a hood. PCR reactions were carried out in an ABI9700 thermocycler (PE Applied Biosystems, UK). PCR samples were injected



onto an ABI PRISM 310 genetic analyser and analysed using the Genemapper analysis software version 3.5 (PE Applied Biosystems, UK). Genescan-500 ROX size standard (PE Applied Biosystems, UK) was used for sizing of labelled PCR products.

#### **A.7 Materials and solutions for a-CGH**

For Spectral Genomics Constitutional Chip<sup>TM</sup> 2.0 and 3.0 slides the reaction buffers were provided by the same company. The buffers included the spectral HYB buffer I, the spectral HYB buffer II, the Spectral labelling buffer and deionized water. Twenty (20) x Random primer solution, EDTA buffer and Klenow fragment enzyme were from the Invitrogen. The Bioprime labelling kit and Cy3 and Cy5 were from GE Healthcare.

The reagents used for Array Genomics were the same used for Spectral Genomics.

For the TRL Mermaid 32K arrays the Invitrogen Bioprime labelling kit was used for the DNA labelling. Cot-1 and Herring sperm DNA were provided from Invitrogen whereas Cy3 and Cy5 were provided from GE healthcare.

For BlueGnome Cytochip arrays the reagents were provided from the company (BlueGnome fluorescent labelling kit). The kit included a reaction buffer, nuclease free H<sub>2</sub>O, Primer solution, a dNTP mix, Cy3 dCTP, Cy5 dCTP, Klenow fragment, sonicated herring sperm DNA and hybridisation buffer (10% dextran sulphate). Human Cot-1 DNA was from Invitrogen.

#### **A.8 Materials and solutions for FISH**

DNA probes were all provided from Vysis UK and FISH solutions were provided from Sigma UK. A dissecting microscope (Olympus) was used for slide preparation as well as for embryo handling.

Solutions for slide preparation and pre-treatment included glass slides (VWR), 10% poly-l-lysine solution, fix solution (three volumes of methanol and one volume of glacial acetic acid), 70% acetic acid, PBS, fresh spreading solution (8.9ml H<sub>2</sub>O, 1ml 1% Tween20 and 100µl 1M HCl), 70%, 90% and 100% ethanol, pepsin solution (1N HCl and 10mg/ml pepsin) and 1% paraformaldehyde/PBS.

For hybridisation, the probe mix apart from the probes consisted of chromosome enumeration probe (CEP) buffer for centromeric probes or locus specific identifier (LSI) buffer. Other solutions and materials used were denaturation mix (70% formamide/2xSSC), 70% 90% and 100% ethanol, 13mm coverslips and Rubber cement.

For post hybridisation washes solutions used were 50% formamide/2xSSC (or 2xSSC/60% formamide in case of co-hybridisation), 2xSSC, 4xSSC/0.05% Tween20 70%, 90% and 100% ethanol and Vectorshield antifade medium counterstain (1.25ng/ml 4', 6-diaminidino 2 phenylindole (DAPI))

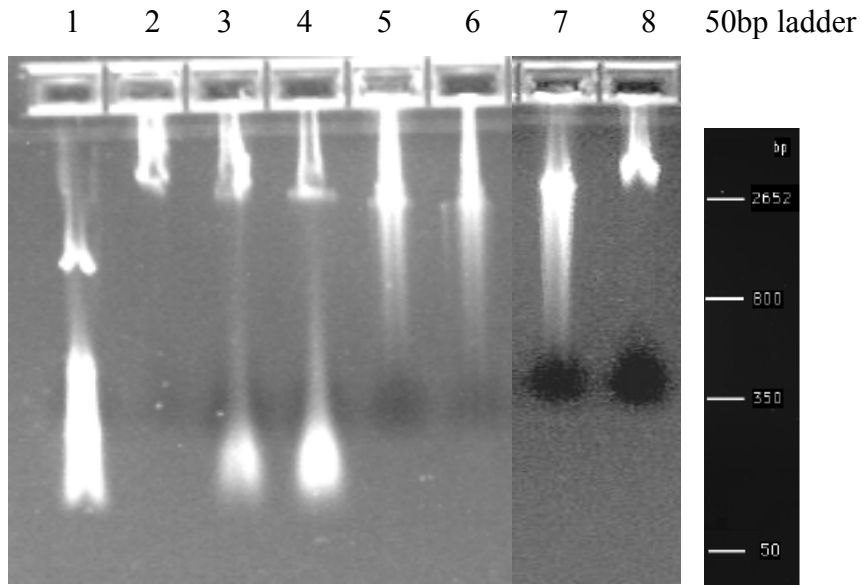
Fluorescent microscopy was achieved with the use of a Zeiss Axioskop microscope with chroma multi-band pass TRITC/FICT/DAPI filter and single Spectrum-Aqua filter. The same microscope with a built in Photometrics KAF 1400 cooled CCD (charged coupled device) camera was used to capture FISH images. SmartCapture software from Vysis was used to control the image capturing. The analysis was done with the utility of special computer software (Digital Scientific, UK), that converted fluorescent intensities into a red, green or aqua colour for each signal.

Primer name	Sequence	Label
<b>Chromosome 5</b>		
<i>D5S1965 -For</i>	TGTCCCCTTGATAAAAATTACTGCG	FAM
<i>D5S1965 -Rev</i>	GTGTCTGGGATTTCTACGCAATG	-
<i>D5S2065-For</i>	CAGCCTCATTGTTTATTGACAG	FAM
<i>D5S2065-Rev</i>	AATGGCATAGTTTTGGCTC	-
<i>D5S656-For</i>	GCTAAGAAAATACGACAACTAAATG	FAM
<i>D5S656-Rev</i>	CATAATAAACTGATGTTGACACAC	-
<i>D5S346-For</i>	ACTCACTCTAGTGATAAATCGGG	FAM
<i>D5S346-Rev</i>	AGCAGATAAGACAGTATTACTAGTT	-
<b>Chromosome 7</b>		
<i>p.Phe508del-For</i>	GTTTTCTTGATTATGCCTGGCA	FAM
<i>p.Phe508del-Rev</i>	GTTGGCATGCTTTGATGACGCTTC	-
<i>IVS8CA-For</i>	AAATCTATCTCATGTTAATGCTGAAGA	NED
<i>IVS8CA-Rev</i>	ACTAAGATATTTGCCATTATCAAGTT	-
<i>IVS17TA-For</i>	TGTCACCTCTTCATACTCATATTGG	NED
<i>IVS17TA-Rev</i>	AACTTACCGACAAGAGGAACTCTG	-
<b>Chromosome 19</b>		
<i>APOC2-For</i>	GGCTACATAGCGAGACTCCATCTCC	FAM
<i>APOC2-Rev</i>	GGGAGAGGGCAAAGATCGATAAAGC	-
<i>DMPK-For</i>	CTTCCCAGGCCTGCAGTTTGCCCATC	VIC
<i>DMPK-Rev</i>	GAACGGGGCTCGAAGGGTCCTTGTAGC	-
<i>D19S112-For</i>	GCCAGCCATTCAGTCATTTGAAG	NED
<i>D19S112-Rev</i>	CTGAAAGACACGTCACACTGGT	-
<b>Chromosomes X, Y</b>		
<i>Amelogenin-For</i>	CCCTGGGCTCTGTAAAGAATAGTC	FAM
<i>Amelogenin-Rev</i>	ATCAGAAGCTTAAACTGGGAAGCTG	-
<i>Amelogenin-Rev</i>	ATCAGAGCTTAAACTGGGAAGCT	-

**Table A1: PCR primer sequences.** This table presents all the sequences of the forward and reverse primers used for all the projects. The forward primers were labelled with FAM, NED or VIC.

### A.9 Agarose gel pictures of MDA products

The following figures illustrate how MDA products from single cells appear on agarose gels and how they are compared to gDNA, MDA products from gDNA and MDA products following DNA digestion by ultrasound.



**Figure A1: Agarose gel electrophoresis of MDA products.** This figure shows the result of 1.2% agarose gel electrophoresis of MDA products Lane 1: 50bp Ladder (Invitrogen, USA), lane 2: gDNA, lane 3: single cell T21 MDA product after ultrasound for 10 seconds, lane 4: single cell T13 MDA product after ultrasound for 10 seconds, lane 5: single cell T21 MDA product without digestion, lane 6: single cell T13 MDA product without digestion, lane 7: MDA product of T21 DNA, Lane 8: gDNA.

#### **A.10 Publications and abstracts arising from this thesis**

**Glentis S**, Craft I, Thornhill A, Harper CJ. (2006) DNA microarray techniques review *Rev of clinical pharmacol and pharmacokinet* **15(2)**: 1-7

**Glentis S**, SenGupta S, Thornhill A, Craft I, Carter L, Rickman CJ, Harper JC. (2006) Multiple displacement amplification as a method of whole genome amplification for preimplantation genetic diagnosis. *Hum. Reprod.*, **21 (1): Suppl.** i40

**Glentis S**, Wang R, SenGupta S, Craft I, Thornhill A, Palmer R, Gibbons B, Harper JC. (2007). Molecular and cytogenetic analysis on multiple displacement amplification (MDA) products from single cells for Preimplantation Genetic Diagnosis (PGD). *EJHG* **15 (suppl 1)**: 117

**Glentis S**, Wang R, Suri S, Palmer R, Sengupta S, Craft I, Gibbons B, Jauniaux E, Harper JC. (2008) Array CGH on coelomic fluid samples *Prenat Diagn* **28**: S32

Mamas T, O'Neill H, **Glentis S**, Harper JC, SenGupta S. (2008) Investigation of single cell lysis and whole genome amplification methods for suitability for haplotyping and microarray comparative genomic hybridisation. *IfWH* 3rd Annual meeting

**Glentis S**, SenGupta S, Thornhill A, Wang R, Craft I, Harper JC. (2009) Molecular comparison of single cell MDA products derived from different cell types *Reprod Biomed Online*: Accepted for publication